July 1, 1993

1991 RESEARCH PROJECT ABSTRACT
FOR THE PERIOD ENDING JUNE 30, 1993
This project was supported by the Minnesota Future Resources Fund (MS 116P.13)

TITLE	Genetic Gamefish Growth Studies
PROGRAM SUBMANAGER	Dr. Perry B. Hackett
ORGANIZATION	University of Minnesota, Twin Cities campus
LEGAL CITATION	M.L. 91, Ch 254, Art. 1, Sec. 14, Subd. 8(b)
APPROPRIATION AMOUNT	$240,000

STATEMENT OF OBJECTIVES

1) To analyze fish hatched from embryos injected with growth hormone genes for the presence and expression of the transgenic elements.
2) To breed second generations of transgenic fish and analyze them for the presence of transgenic growth hormone gene.
3) To develop new expression vectors containing fish growth hormone genes and fish genetic regulatory elements.

RESULTS

We have successfully kept alive several of the proven transgenic, growth-enhanced fish that were produced between 1988-1990. These fish were examined for accelerated growth, morphology, and ability to produce offspring. Of the more than 10,000 transgenic northern pike, walleye, rainbow trout, and Atlantic salmon, less than 200 founders survived the continued stress that occurred when the fish were moved around the state of Minnesota from one indoor facility to another. This was necessary because the construction of an indoor Transgenic Fish Facility could not be constructed on time. Additionally, sporadic fluctuations in temperature and chlorine levels at some facilities resulted in mortality. These problems will be alleviated with the merger of the Transgenic Fish facility with the University of Minnesota Aquaculture Facility (the other part of this project grant) that should open in Winter 1993. Nevertheless, we have a few founders left, and have second generation northern pike, rainbow trout, and Atlantic salmon. Additionally, we have isolated an additional fish growth hormone gene, characterized further genetic regulatory elements used to construct recombinant DNAs that could express the extra growth hormone genes. These studies show the effects of different regulatory elements in different transgenic fish and cultured fish cells. In sum, Minnesota now has the richest source of growth enhanced fish in the United States. What is now required is evaluation of the potential environmental impact of these fish and their exploitation by Minnesota aquaculturists.

PROJECT RESULTS USE AND DISSEMINATION

The work supported by this grant resulted in 8 research papers, one review, and nearly a dozen symposia abstracts, four of which were international. The following papers have received acclaim worldwide:

PEER REVIEWED PAPERS

Liu, Z. et al. (1991). Importance of the CArG box in regulation of b-actin encoding genes. Gene 108: 211-217.
Gross, M.L. et al. (1992) Growth evaluation of northern pike (Esox lucius) injected with growth hormone genes. Aquaculture 103: 253-273.
Schneider, J.F. et al. (1992) Molecular cloning of the cDNA for northern pike (Esox lucius) growth hormone gene. Molecular Marine Biology and Biotechnology 1: 106-112.
He, L. et al. (1992). Characterization of AluI repeats of zebrafish (Bracydanio rerio). Molecular Marine Biology and Biotechnology 1: 125-135.
Moav, B. et al. (1992). Selection of promoters for transgenic fish. Molecular Marine Biology and Biotechnology 1: 338-345.
Moav, B. et al. (1993). Regulation of early expression of transgenes in developing fish. Transgenic Research 1: 153-161.
Ivics, Z., et al. (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Molecular Marine Biology and Biotechnology 2: 162-173.
Caldovic, L. et al. (1993). Improved expression vectors for transgenic fish. (in preparation for Molecular Marine Biology and Biotechnology).
Hackett, P.B. (1993). The molecular biology of transgenic fish IN Biochemistry and Molecular Biology of Fishes Vol. 2 (Hochachka and Mommsen, eds.) Elsivier Scientific Publishers.

LCMR Final Status Report - Detailed for Peer Review-Research

GENETIC GAMEFISH STUDIES

[M.L. 91, Ch 254, Art. 1, Sec. 14, Subd. 8(b)]

Principal Investigators

Dr. Perry B. Hackett, Department of Genetics and Cell Biology
Dr. Anthony J. Faras, Institute of Human Genetics

Program Manager
Dr. Ira Adelman, Dept. of Fisheries and Wildlife

University of Minnesota, Twin Cities campus

1 July 1993

July 1, 1993

1991 RESEARCH PROJECT ABSTRACT

FOR THE PERIOD ENDING JUNE 30, 1993

This project was supported by the Minnesota Future Resources Fund (MS 116P.13)

TITLE	Genetic Gamefish Growth Studies
PROGRAM SUBMANAGER	Dr. Perry B. Hackett
ORGANIZATION	University of Minnesota, Twin Cities campus
LEGAL CITATION	M.L. 91, Ch 254, Art. 1, Sec. 14, Subd. 8(b)
APPROPRIATION AMOUNT	$240,000

STATEMENT OF OBJECTIVES

1) To analyze fish hatched from embryos injected with growth hormone genes for the presence and expression of the transgenic elements.
2) To breed second generations of transgenic fish and analyze them for the presence of transgenic growth hormone gene.
3) To develop new expression vectors containing fish growth hormone genes and fish genetic regulatory elements.

RESULTS

We have successfully kept alive several of the proven transgenic, growth-enhanced fish that were produced between 1988-1990. These fish were examined for accelerated growth, morphology, and ability to produce offspring. Of the more than 10,000 transgenic northern pike, walleye, rainbow trout, and Atlantic salmon, less than 200 founders survived the continued stress that occurred when the fish were moved around the state of Minnesota from one indoor facility to another. This was necessary because the construction of an indoor Transgenic Fish Facility could not be constructed on time. Additionally, sporadic fluctuations in temperature and chlorine levels at some facilities resulted in mortality. These problems will be alleviated with the merger of the Transgenic Fish facility with the University of Minnesota Aquaculture Facility (the other part of this project grant) that should open in Winter 1993. Nevertheless, we have a few founders left, and have second generation northern pike, rainbow trout, and Atlantic salmon. Additionally, we have isolated an additional fish growth hormone gene, characterized further genetic regulatory elements used to construct recombinant DNAs that could express the extra growth hormone genes. These studies show the effects of different regulatory elements in different transgenic fish and cultured fish cells. In sum, Minnesota now has the richest source of growth enhanced fish in the United States. What is now required is evaluation of the potential environmental impact of these fish and their exploitation by Minnesota aquaculturists.

PROJECT RESULTS USE AND DISSEMINATION

The work supported by this grant resulted in 8 research papers, one review, and nearly a dozen symposia abstracts, four of which were international. The following papers have received acclaim worldwide:

Liu, Z. et al. (1991). Importance of the *CArG* box in regulation of b-actin encoding genes. Gene 108: 211-217.

Gross, M.L. et al. (1992) Growth evaluation of northern pike (*Esox lucius*) injected with growth hormone genes. Aquaculture 103: 253-273.

Schneider, J.F. et al. (1992) Molecular cloning of the cDNA for northern pike (*Esox lucius*) growth hormone gene. Molecular Marine Biology and Biotechnology 1: 106-112.

He, L. et al. (1992). Characterization of *Alu*I repeats of zebrafish (*Bracydanio rerio*). Molecular Marine Biology and Biotechnology 1: 125-135.

Moav, B. et al. (1992). Selection of promoters for transgenic fish. Molecular Marine Biology and Biotechnology 1: 338-345.

Moav, B. et al. (1993). Regulation of early expression of transgenes in developing fish. Transgenic Research 1: 153-161.

Ivics, Z., et al. (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Molecular Marine Biology and Biotechnology 2: 162-173.

Caldovic, L. et al. (1993). Improved expression vectors for transgenic fish. (in preparation for Molecular Marine Biology and Biotechnology).

Hackett, P.B. (1993). The molecular biology of transgenic fish IN *Biochemistry and Molecular Biology of Fishes* Vol. 2 (Hochachka and Mommsen, eds.) Elsivier Scientific Publishers.

Introduction

The initial drive for transgenic fish came from attempts to enhance production of economically-important fish. The United States Department of Agriculture (10), The Minnesota Department of Agriculture (22) and other world-wide agencies have observed a leveling off of world-wide fish production. With an increasing population, there will be considerable pressure to increase fish production. In 1992 there were less than 20 producers of food fish in Minnesota with net sales of a little over $200,000 (22). This is an amazing statistic since the Chicago Fish House, a fish wholesaler for the Chicago metropolitan areas imports about $50,000,000 in fish annually from Norway. In 1986 four professors at the University of Minnesota [Dr. Kevin Guise (Department of Animal Science), Anne Kapuscinski (Dept. of Fisheries and Wildlife), Anthony Faras (Institute of Human Genetics) and Perry Hackett (Dept. of Genetics and Cell Biology) asked two questions, why is this money going outside the United States and why should it not go to Minnesota? The answer was that 1) Minnesota does not currently have facilities to grow fish, 2) the state is not particularly suitable for normal fish aquaculture, and 3) the problem is relatively new and has not been publicly acknowledged. We reasoned several years ago that application of modern genetic technology to aquaculture offered a possible solution to some shortages of fish and could provide the state of Minnesota with an additional economic resource. Our goal was to use genetic engineering (also known as transgenic technology) to improve the characteristics of several species of fish so as to increase their commercial value.

The current project was a successor to the first. It was conducted originally by Professors Guise, Faras and Hackett but, owing to Dr. Guise's premature death soon after initiation of the grant, was finished by the Faras and Hackett laboratories. Table 1 (next page) shows a list of personnel that were involved with the second phase of this project.

Table I

MINNESOTA TRANSGENIC FISH GROUP
1990-1993

Principle Investigators	Postdoctoral Fellows and Students
Prof. Perry Hackett[1,2]	Dr. John Schneider
Prof. Anthony Faras[2,3]	Dr. Zhanjiang Liu
Prof. Kevin Guise[+]	Dr. Zsuzsanna Izsvak
Prof. Anne Kapuscinski[4]	Dr. Zoltan Ivics
	Dr. Gonzalo Martinez
	Mark Gross
	Ling He
	Jeffery Essner
	Ljubica Caldovic
	Scott Fahrenkru
	Joachim Breuer
Collaborators and Visitors	**Research Staff**
Dr. Zouyan Zhu (P.R. China)	Mark Gross
Dr. Boaz Moav (Israel)	Mark Hovie
Dr. Naomi Moav (Israel)	Steve Myster
Dr. Carmen Alvarez (Spain)	Russell Essner
Dr. Choy Hew (Canada)	Eric Sell
Dr. Brian McKeown (Canada)	

1. Department of Genetics and Cell Biology, University of Minnesota, St. Paul
2. Institute of Human Genetics, University of Minnesota, Minneapolis
3. Department of Microbiology, University of Minnesota, Minneapolis
4. Department of Fisheries and Wildlife, University of Minnesota, St. Paul

[+]deceased 2/91

Dr. Kapuscinski, though not a formal collaborator was in the initial collaboration and will be using the fish that were produced and analyzed in this project. Accordingly, she is listed as well. This report is divided into three sections. The first part is an overall

introduction to the study; it provides the rationale for the project as a whole. The second portion is Appendix II - Summary Report which lists the most important results pertaining to fish status. The third portion is Appendix III - Published Reports, the section that contains reprints of the papers that we published throughout the project period. They contain the material for peer review; indeed all have undergone rigorous peer review before publication.

Introduction
Transgenic Fish in Aquaculture

What can transgenics offer to aquaculture? Presently there are four areas of potential application: growth enhancement, cold tolerance, disease resistance, and phenotypic marking. These are discussed next.

Growth enhancement.

Improving the growth rates of fish was one of the initial motivations for genetically engineering fish based on the findings that mouse size could be significantly enhanced following incorporation of a rat growth hormone gene into the mouse genome (25). Three aspects of fish growth could be improved for economic purposes: 1) initial growth rate such that they reach maturation earlier, 2) enhanced growth rate as adults to provide larger fish for market, and 3) fish with improved feed effic-
iciency. In summary, enhanced fish growth rates show considerable promise. Reports up to 1991 demonstrated that carp, salmon, northern pike, loach, trout, and catfish could be transformed with a variety of growth hormones under the control of different promoters to produce fish with growth enhancements of up to 100% that of controls (1,5,9,29,30). Several problems existed with these studies. First, many of the transgenic constructs or growth hormone genes are publicly unacceptable. The use of viral or heavy metal-inducible promoters (*e.g.*, RSV LTR, MT) leads to associations with disease and or metal toxicity ("leaded fish"). A second problem is how applicable the results obtained under indoor lab conditions will be to outdoor rearing. In nearly all cases except those in China, Russia and Israel, transgenic fish are raised indoors under artificial conditions that normally are not used for commercial aquaculture and not optimal for fish rearing to maturity. Consequently, growth rates of transgenic fish with GH genes may vary considerably depending on the facilities available to different research groups. Indeed, the effects of the facility may be greater than those of species variation, enhancer/promoter choice, or source of growth hormone gene/cDNA.

A quantum level leap was recently reported by Choi Hew's group in Toronto, Canada (5). Atlantic salmon embryos were microinjected with an ocean pout-enhancer-promoter/chinook salmon-GH cDNA construct to yield transgenic fish that were two to six-fold larger than controls (one fish that was 13 times the average of fish raised from non-microinjected eggs). The fish for this study were selected on the basis of their size and presence of the transgene in their nuclei. Interestingly, the largest fish died prematurely. The work has been extended to Pacific salmon and Chinook salmon, with the same general results. Also of interest is the all-fish expression vector that was employed (5). The opAFP promoter is not particularly strong and is much weaker than the carp β-actin enhancer, MT promoters and RSV LTR's that have been used by others (e.g., 2). These results suggest that either the strongest consitutively-acting promoters may not always be the best for achieving particular phenotypes, that the Hew group has a particularly fine environment for raising contained fish, or both.

2. Cold Tolerance

It is cold in Minnesota, and in Canada. To develop an aquaculture industry in such harsh climates, fish with tolerances against cold would be useful and economically

beneficial. These considerations led a Canadian consortium of labs, much like ours at the University of Minnesota, to collaborate on the isolation, cloning and transgenic expression of antifreeze protein (AFP) genes found in cold water fish such as the winter flounder (wf), ocean pout (op), and sea raven (sr) that permit survival in freezing seawater, -1.8°C (7,26). The genes and their regulatory elements show evolutionary homology (3,12). Atlantic salmon with transgenic AFP genes have been produced (26). 3% of the zygotes microinjected showed expression of the gene. When two of these founders were backcrossed with control fish, 40% of the offspring carried the gene as detected by polymerase chain reaction (PCR) and 53% of the F2 generation produced by backcrossing F1's with wild type fish showed evidence of the gene. However, expression of the gene was not reported and the fish do not show a measurably increased tolerance to cold (C. Hew, pers. comm.).

3. Disease resistance

Diseases, especially viral diseases can be disastrous to a commercial aquaculture facility. Infectious hematopoetic necrosis virus (IHNV) is a fish rhabodovirus that has caused extensive mortality in northwest Pacific trout and salmon hatcheries. Retroviruses have been found in walleye (21) and northern pike (J. Casey, pers. comm.), though no diseases have been associated with their presence as yet. Two approaches have been undertaken by Leong and her collaborators to find therapeutic measures that will improve virus resistance. The 66 kD glycoprotein (G) gene of the virus has been cloned and expressed in a baculovirus vector (18) to provide large amounts of the protein for passive inoculation by dipping the fish into water containing the G-protein in order to stimulate antibody production against the antigen and thereby achieve immunity (J. Leong, pers. comm.). The viral G-protein has also been cloned as a fusion protein in an *E. coli* expression vector for epitope mapping and protein characterization (28). However, effective immunity on a vast scale might be achieved by transfer of the G-protein gene into the teleost genome for expression in all cell membranes to prevent infection. Accordingly, the Hackett lab cloned both the complete gene and a partial gene into the carp β-actin expression vector for delivery into trout and salmon eggs. These experiments could prove to be more valuable to the aquaculture industry than growth enhancement and cold tolerance. Moreover, from an ecological perspective, the inherent danger of such a genetically engineered fish is relatively minimal. An alternative strategy for disease resistance would be to incorporate genes into the piscine genome that would nullify viral activity once the virus penetrated cellular membranes. By employing anti-sense gene constructs (16,27), wherein a critical viral gene is oriented backwards to a constitutive promoter, an RNA would exist in cells that could hybridize and completely block expression of the invading viral gene.

4. Phenotypic markers

Development of a quick visual screening procedure for transgenic fish would be beneficial in two ways. First, pigmentation of the fish would be convenient and the tyrosinase gene responsible for melanin formation is an obvious candidate though a variety of coloration mutations are known in the zebrafish (Westerfield, pers. comm.). Work on identifying the zebrafish tyrosinase gene, and others, is ongoing. Second, phenotypic marking would permit easier inspection of fish outside transgenic fish rearing facilities for detection of accidental release of genetically engineered animals. Readily detected transgenic fish might help ameliorate environmental concerns (17). The first report of pigment marking of albino zebrafish employed production of germ-line chimeras using cell transplants from genetically pigmented embryos to embryos from pseudo-albino parents (20). The technique is based on previous work done in mice where HPRT-deficient mouse embryos were produced via embryonal stem cell transplantation (13,17). Thus, transfer of whole cells with complete chromosomal complements is an alternative to single gene transfer.

Genetic Engineering of Fish in Minnesota

Our first goal was growth enhancement of commercially valuable fish, with the understanding that if we succeeded in this area, we could continue improvement of fish species for Minnesota aquaculture. From 1988 to 1990 we produced the first genetically engineered fish in Minnesota. More than 60,000 embryos of walleye, northern pike and rainbow trout were microinjected with DNA constructs that contained a growth hormone gene and required genetic switches to ensure expression of the transgene. From the thousands of embryos that were microinjected, only about one thousand fish survived to adulthood. The low survival was due to many factors including constant stress of moving from one fisheries facility to another in various regions of the state, the lack of knowledge of indoor rearing of wild game fish (generally they are raised in outdoor facilities), the natural cannibalism practiced by the fish at early stages, and possible lowered fitness of growth-enhanced, transgenic fish.

In 1991 a subgroup of the original collaboration, Profs. Kevin Guise, Perry Hackett and Tony Faras, obtained further funding from the LCMR to continue the project. The project was lumped together with a project headed by Prof. Ira Adelman (Dept. of Fisheries and Wildlife) to develop an Aquaculture Facility at the University of Minnesota. Six months after the initiation of the grant, Prof. Guise died and the project was continued by Drs. Hackett and Faras.

Specific Project Goals

1. Analyze existing potentially transgenic fish from the earlier study to determine transgenic status.
2. Breed transgenic fish at maturity.
3. Analyze offspring of transgenic fish for their transgenic status.
4. Determine growth enhancement of second generation transgenic fish. and identify potential transgenic broodstock.
5. Isolate new piscine growth hormone genes.
6. Construct new fish expression vectors for delivery of growth hormone genes into embryos.
7. Produce and analyze new transgenic fish for transgenic status.
8. Initiate growth analysis of new transgenic fish.

RESULTS

GOAL 1: Analysis of transgenic fish.

Although the number of surviving founder fish is low, many are transgenic. Only one or two actively expressing fish are required to serve as broodstock for future generations. We had two types of vector that were injected into the fish, i) RSV/bGH [Rous sarcoma virus enhancer-promoter directing the synthesis of bovine growth hormone mRNA] and ii) β-act/csGH [fish β-actin promoter directing the synthesis of chinook salmon growth hormone gene]. Initially we used the RSV/bGH construct for test purposes in the northern pike and then switched to the β-act/csGH construct for northern pike, walleye, rainbow trout and Atlantic salmon.

About 10,000 northern pike embryos were injected with the two transgenic constructs. Of several thousand embryos injected with the RSV/bGH construct 1218 were examined by radioimmunoassay and 36 (3%) had elevated levels of bGH in their blood. Of the several thousand embryos that were injected with β-act/csGH, 1398 were screened to yield 88 (6%) with elevated csGH in the blood. This was in the range we expected.

Previous studies that we and others have conducted indicated that mosaicism was common, i.e., that the transgenic DNA integrated into the fish chromosomes after the initial cleavages yielding fish that had the transgene in some tissues but not others. This determination came from Southern blotting of various tissues (muscle, fin, blood, kidney, spleen, heart, brain). The bottom line was thus, by using PCR techniques on small samples of fin from the experimental fish, we could determine whether or not the transgenic construct was present in the fin but not necessarily in the most important tissue, the gonads. Presence in the gonads is important for passage of the trait to offspring. To further determine our abilities to get the transgenic DNA into fish chromosomes, we sacrificed a sample of the fish and did Southern blotting analysis of tissues. 30% of the fish had the transgene in one of the tissues analyzed, but only about 12% had the gene in fin tissue. Thus, a positive signal in the fin samples represented only about 40% of the transgenic fish.

Why did only 3-6% of the fish show elevated levels of transgenic GH when 30% of the fish had the transgene in one or more tissues? The answer is not known. The best speculation, that is fairly well founded on other systems, is that expression of the transgenes is dependent not only on the accompanying genetic regulatory elements, but also controlling sequences in the fish chromatin near the site of integration of the construct. There are about 2,000,000,000 potential sites of entry of the transgenic material, and the regulatory units around these sites will almost certainly play a role in transgene expression. We are currently working on this problem. Likewise, the problem of mosaicism is widespread in every laboratory world-wide that is attempting to make transgenic fish. Screening transgenic fish would be much easier, and the results of the procedure far more predictable, if mosaicism were reduced. Accordingly, we have initiated studies to improve the rate of early integration of transgenes into fish (15).

GOAL 2: <u>Breeding of transgenic fish</u>

For breeding, we needed to maintain the fish until they reached sexual maturity. This required warehousing the fish for 2-4 years. This was challenging in two regards. First, the fish had to be reared indoors. Since the University of Minnesota Department of Fisheries and Wildlife does not have adequate facilities for rearing of so many adult fish, we had to use DNR facilities in St. Paul as well as NSP facilities in Cohasset and New London. These indoor tanks were needed for different purposes at different times of year necessitating movement of the fish at least twice a year. This translocation of the fish induced considerable stress and subsequent mortality. Moreover, in the Cohasset and New London facilities there were periodic breakdowns in water quality (temperature and chlorination) which resulted in occasional fish kills. Consequently, we realized that the only way to improve the chances of keeping the fish alive was to move them to University of Minnesota tanks. This necessitated culling most of the control fish (fish from eggs that were not microinjected with GH constructs; eggs were from the same batches as those that were microinjected with transgenic GH genes) and discarding those fish that did not show any evidence of transgenic GH in either blood serum or fin samples. The second challenge was to get the fish to mate indoors, a problem which has proved to be very difficult in the wild fish. Below we report the survival of fish and the breeding we have done with the survivors.

a) Northern pike:

<u>1988 Founder stock (RSV/bGH)</u>: 36 founder fish remain from the 1988 founder stock (7 males and 29 females), including all five previously identified as positive for the transgenic Growth Hormone (GH) gene construct. 24 control siblings are also alive. Growth studies have indicated an enhancement effect of the extra growth hormone gene in some cases. One two-year old (1990) cross exists as of 6/93, however only 2/25 remaining siblings contain an extra growth hormone gene (other positive siblings died).

These positive fish have not demonstrated enhanced growth, however rearing conditions have been much less than ideal. In 1991, 3/8 crosses of the 1988 stock were positive for the transgenic GH gene by Southern blot analysis. Only 12 fish remain from these crosses. Many of the remaining 1988 founder fish spawned during the month of May 1993: 9 males were mated with 15 females to yield 23 crosses (2 of these are control sibling crosses). As of June, 1993, 18 crosses have had various degrees of success hatching. These crosses will be screened for transmittance of transgene in July, 1993.

1989 Founder stock (β-act/csGH): In April 1991 due to limited space on the St. Paul campus, these fish were split and moved to hatcheries in Cohasset and New London, MN. The Cohasset fish all died from a facility malfunction. The New London fish went through strong negative selection due to health problems (well water on the St. Paul campus is from an aquifer and thus somewhat sterile; whereas the NL water was taken from the bottom of a outdoor pond and thus contained all of the parasites to which a fish in the wild may succumb, winter water temperatures was only 39`F). Only the fittest fish survived, which, interestingly, did not include the known transgenic founder fish. Only 54 of the ~200 fish (>50% known to be transgenic) that were moved to NL returned to the St. Paul Campus, and only 2 of these were known to carry transgenic GH gene from blood and tissue assays by Southern blotting. As of June, 1993 only 33 fish survive, with only one of the known transgenic founders being among them. However, this female is the fourth largest amongst its siblings. The other 32 are potential mosaics; they are not positive in fin and blood samples but may be positive in their gonads. Consequently, these fish are being kept until gametes can be obtained and screened for transgenic GH gene. The remaining 1989 founders did not produce gametes in 1993.

b) Rainbow trout:

1989 Founder stock (RSV/bGH): No founders have survived. 25 crosses were made in 1990. 6 of 14 crosses tested at the embryo stage were found, by Southern blotting of DNA from selected samples of embryos, to be positive for extra bGH genes. However, when checked again after survival to the fingerling stage, only two crosses still had positive offspring, as of 6/93 there are 45 siblings alive of which only 3 possess transgenes. There is no apparent growth enhancement in these fish.

1990 Founder stock (β-act/csGH): The health of these fish deteriorated as the 1993 spawning season approached. 39 fish died during this stressful period. The reason for these deaths is not understood, but it also affected gamete quality in the remaining living fish. Only 4 females gave eggs of sufficient quality to be fertilized during February and March of 1993. Different males were used to fertilize these eggs to yield 9 crosses. 3 of these hatched and are presently being screened for transgene transmittance. Two of these crosses have approximately 1000 siblings each, the third cross only has 26 siblings. Of the remaining 61 founder stock, 12 fish are still alive that are known to be positive for extra csGH genes. Fortunately, all of the fish known to transmit the extra csGH genes are still alive. The other founders are all potentially mosaic and thus are potentially capable of transgene transmittance (when these fish eventually breed we will be able to determine if transmittance is possible). All control founder siblings were sacrificed due to space limitations thus destroying any ability to determine growth enhancement in the founder stock. There are three positive crosses from the 1992 mating season that were screened for transgene transmittance (about 400 fish) in May 1993. Only one of these appears to have any positive offspring remaining; since the frequency is about 1% for transmittance, the germ line is apparently mosaic.

c) Atlantic salmon:

1990 Founder stock (β-act/csGH): As of June, 1993, 118 founder fish are surviving; 5 of these are known to be positive for transgenic csGH gene within their fin tissue. The remaining fish are potentially mosaic, and thus have a chance of having transgenic gametes. 20 early-maturing female fish were bred in late 1992: 21 crosses were made of which 17 were successfully fertilized. Unfortunately, most of the embryos died shortly before hatching. There are about 130 fish remaining alive from 8 of these crosses. The low survivability might be due to the early maturation of the gametes, a full year early. Whether the transgene was passed on in these crosses is unknown as of yet. 25 males produced gametes, however, analysis of the sperm DNA showed only 1 possessed the transgene. The growth performance of the Atlantic salmon founder fish is impossible to evaluate since all control siblings have been sacrificed due to space limitations. Nearly all founders should spawn in 1994, and the gametes are expected to be of high quality.

d) Walleye:

1990 Founder stock (β-act/csGH): All have died. No progeny were obtained before death..

GOAL 3: Analysis of F1 generation fish for transgenic GH genes. The fish are too young for this analysis without damage to the individual. If transgenic, all tissues will have the genes and thus PCR analysis of fin clips will be sufficient for analysis. This analysis will be initiated once the F1 offspring have been moved into the University of Minnesota Aquaculture and Transgenic Fish Facility. Then stocking densities and consequential stress will be reduced which should permit better rates of healing of clipped fish.

GOAL 4: Analysis of F1 generation fish for growth enhancement and identification of broodstock As noted above, we had to cull most of the control fish; thus adequate controls do not exist. Moreover, currently we do not have any space to rear control fish. All of our efforts are to keep transgenic fish alive and to cultivate their offspring. The fish are too young for this analysis without damage to the individual. As noted above in the general discussion of growth enhancement of transgenic fish, we can do the studies, but there will be effects of the containment that will compromise the results somewhat. In the future, the fully transgenic F1 and F2 generation fish can be compared for growth enhancement using the same techniques as discussed in the Gross et al. paper (see Appendix III).

GOAL 5: Isolation of more fish GH genes. The Schneider et al. paper in Appendix III reports on our isolation and characterization of a northern pike cDNA copy of a GH gene. The pike GH gene is comprised of 209 amino residues, including a signal sequence of 22 amino acids. The gene is closely related to salmon and trout GH genes. Between these species, about 10% of the amino acids are different, but many of these are conservative substitutions.

GOAL 6: Construction of more advanced fish expression vectors. Expression vectors are recombinant DNAs that carry the transgene of interest and the regulatory sequences that determine where, when, and the level at which the transgene will be expressed. All of the vectors reported so far are plasmids that can be replicated to levels of 500-2000 copies per *Escherichia coli* cell. Since *E. coli* can be grown easily to $2\text{-}5 \times 10^9$ cells/ml of culture broth, one milliliter of bacterial cells can produce more than 10^{13} recombinant plasmids/ml. The ability to obtain practically unlimited copies of recombinant DNAs in relatively pure form makes transgenic work possible. Genetic

regulatory elements are sites required for initiation of transcription, termination of transcription, RNA splicing and initiation of protein synthesis. The genetic elements regulating initiation of transcription will determine when, where and extent to which expression will occur. Consequently these signals have been been the focus of considerable study by many labs. Transcriptional termination, the generic term for 3' cleavage and polyadenylation, and splicing are important and necessary. But, since they are generally considered to be ubiquitous there has been far less study of how these elements can be used to regulate gene expression in transgenic animals. The promoter and the enhancer/silencer sites are *cis*-acting; they affect transcription on the same segment of DNA on which they reside. Generally the proximal promoter region of most genes will direct a basal level of transcription that may be enhanced or depressed according to the availability of *trans*-acting protein factors in the particular cell type (*e.g.*, 4,8,14,23,24).

Our molecular dissection of the carp β-actin gene and its transcriptional regulatory sequences has shown that they were more conserved than either the cDNA nucleotide sequence or the amino acid sequence (Liu et al. paper, Moav et al. papers, see Appendix III). Our first vector used the carp β-actin gene enhancer-promoter and first intron of the β-actin gene in concert with the poly(A) cleavage/addition signal from chinook salmon (20'). This vector came in two major types, one with just the proximal promoter and the second with all of the enhancers including an enhancer in the first intron. Since the first exon is non-coding for all vertebrate actin genes so far characterized, the intron actually precedes the transgene insertion site. The salmon GH 3' end was chosen over the β-actin 3' sequence since the latter has a silencer sequence next to the poly(A) signal that reduces expression in muscle cells. Consequently, these expression vectors are constitutively expressed in nearly all tissues. The earliest form of the vectors had a single *Kpn*I site for addition of the desired transgene. Further developments have produced FV-5 and FV-6 fish expression vectors which have a complete polycloning sequence for insertion of a greater varieties of DNA (Appendix III; Caldovic, in preparation). We also tried a vector with repeating DNA sequences flanking the transgene in order to accelerate integration but the vector was unsuccessful (see He et al., Appendix III). Another avenue to accelerate integration using co-injection of integrase proteins with the transgenic constructs does appear to be a method that could prove to be of immense value in fish as well as other biological organisms (see the Ivics et al. paper, Appendix III).

We have tried alternative procedures for delivery of transgenic DNA (Izsvak et al., unpublished experiments) including lipofection. However, we conclude that with the relative rapidity of microinjection and the rates of success already achieved that have stressed our abilities to analyze the offspring, microinjection is still a reasonable method for delivery of transgenic material. The following can be concluded from our accumulated experiments: 1) Embryonic survival for most species following microinjection is 80-90% that of uninjected controls when less than 80 pg ($<10^7$ copies of transgenic DNA) were transferred; the outcome of most experiments depends on that elusive characteristic, *egg quality*. 2) Microinjection generally leads to about 50-80% of the eggs being able to express the transgenic DNA shortly after gene transfer but the persistence of high levels of expression is not maintained over the generation time of the fish and this remains a problem. 3) Nearly all (90-99%) of fish that have integrated transgenes will be mosaic for its presence and/or level of expression, suggesting that integration of the injected DNA was not efficiently happening in the 1-cell stage of embryonic development. These relatively high rates of mosaicism and low transgenic level are compensated by the large number of eggs that can be microinjected by the accomplished investigator. After training, rates of injection of between 50 and 1000 eggs/hr, depending on the type of eggs, the logistical support, and the accuracy of

injection, can be achieved. Nevertheless, fish of commercial importance such as salmon, trout, carp catfish, northern pike, and walleye spawn once per year and deliver 800 to 100,000 eggs/spawn. These eggs undergo their first cleavages in 30 min to 15 hours (6). Although eggs and sperm can be saved for days by refrigeration, there is relatively little time for injection of massive numbers of embryos if egg and milt quality is to be preserved. Injection rates of 1000 eggs/hr though amazing are still insufficient when successful production of germ-line transgenic fish is a few percent or less. Consequently, there is a major drive to find more efficient procedures for mass transfer of DNA to thousands of eggs at once.

THE FUTURE

Owing to environmental concerns (17), further treatment of growth enhanced fish will probably be required. In addition to particular rearing facilities such as those at Auburn University, the fish will probably require sterilization to provide added security against escape. Treatments to induce sterility and/or other genetic manipulations could also provide further phenotypic advantages as well as warranties against accidental release. Curiously, the increased mortality the larger transgenic salmon and pike in a couple of growth trials suggests that unbalanced levels of growth hormone may reduce fitness in transgenic fish. If so this would have significance in terms of fears of accidental escape of genetically engineered *superfish.*

References

1. Agellon, L.B., C.J. Emery, J.M. Jones, S.L. Davies, A.D. Dingle, and T.T. Chen. Promotion of rapid growth of rainbow trout (Salmo gairdneri) by a recombinant fish growth hormone. *Can. J. Fish. Aquat. Sci.* 45: 146-151, 1988.
2. Chen, T.T., K. Kight, C.M. Lin, D.A,. Powers, M. HGayat, N. Chatakondi, A.C. Ramboux, P.L. Duncan, and R.A. Dunham. (1993). Expression and inheritance of RSVLTR-rtGH1 complementary DNA in the trangenic common carp, *Cyprinus carpio. Mol. Mar. Biol. Biotech.* 2: 88-95.
3. Davies, P.L., C.L. Hew and G.L. Fletcher. Fish antifreeze proteins: physiology and evolutionary biology. *Can. J. Zool.* 66: 2611-2617, 1988.
4. Du, S.J., Z. Gong, C.H. Tan, G.L. Fletcher, and C.L. Hew. Development of an "all fish" gene cassette for gene transfer in aquaculture. *Mol. Mar. Biol. Biotech.* (In Press), 1992.
5. Du, S.J., Z. Gong, G.L. Fletcher, M.A. shears, M.J. King, D.R. Idler and C.L. Hew. Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *Bio/Technology* 10: 176-181, 1992.
6. Fletcher, G.L. and P.L. Davies. Transgenic fish for aquaculture. *Genetic Engineering* 13: 331-370, 1991.
7. Fletcher, G.L., M.A. Shears, J.J. King, P.L. Davies and C.L. Hew. Evidence of antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). *Canad. J. Fish Aquat. Sci.* 45: 352-357, 1988.

8. Friedenreich, H. and M. Schartl. Transient expression directed by homologous and heterologogous promoter and enhancer sequences in fish cells. *Nuc. Acids Res.* 18: 3299-3305, 1990.
9. Gross, M.L., A.R. Kapuscinski, J.F. Schneider, Z. Liu, N. Moav, B. Moav, S. Myster, C. Hew, K.S. Guise, P.B. Hackett and A.J. Faras. Growth evaluation of northern pike (*Esox lucius*) injected with growth hormone genes. *Aquaculture* 103: 253-273, 1992.
10. Harvey, D. Aquaculture: Situation and outlook report. *USDA Aqua-5* , 19 pp., 1990.
11. Hew, C. Transgenic fish: present status and future directions. *Fish Physiol. Biochem.* 7: 409-413, 1989.
12. Hew, C.L., N.-C. Wang, S. Joshi, G.L. Fletcher,, G.K. Scott, P.H. Hayes, B. Buettner, and P.L. Davies. Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, *Macrozoarces americanus. J. Biol. Chem.* 263: 12049-12055, 1988.
13. Hooper, M., K. Hardy, A. Handyside, S. Hunter, and M. Monk. HPRT-deficient (Lesch-Nyan) mouse embryos derived from germline colonization by cultured cells. *Nature* 326: 292-295, 1987.
14. Hong, Y., C. Winkler, G. Brem and M. Schartl. Development of a heavy metal-inducible fish specific expression vector of gene transfer in vitro and in vivo. *Aquaculture* (In Press), 1992.
15. Ivics, Z., Z. Izsvak, and P.B. Hackett . Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. *Mol. Mar. Biol. Biotech.* 2: 162-173, 1993.
16. Izant, J.G. and H. Weintraub. Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. *Science* 229: 345-351, 1985.
17. Kapuscinski, A.R. and E.M. Hallerman Implications of introduction of transgenic fish into natural ecosystems. *Canad. J. Fisheries and Aquatic* 48: 99-107, 1991.
18. Koener, J.F. and J.C. Leong. Expression of the glycoprotein gene from a fish rhabodovirus by using baculovirus vectors. *J. Virol.* 64: 428-430, 1990.
19. Kuehn, M.R., A. Bradley, E.J. Robertson, and M.J. Evans. A potential animal model for Lesch-Nyan syndrome through introduction of HPRT mutations into mice. *Nature* 326: 295-298, 1987.
20. Lin, S., W. long, J. Chen, and N. Hopkins. Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. *Proc. Natl. Acad. Sci. USA.* 89: 4519-4523., 1992.
21. Martineau, D. et al.. Molecular characterization of a unique retrovirus associated with a fish tumor. J. Virol. 66: 596-599, 1992.
22. *Minnesota AQUAculture: A Report on the Status of Aquaculture in Minnesota.* Minnesota Department of Agriculture. pp. 46, 1992.
23. Moav, B., Z. Liu, L.D. Caldovic, M.L. Gross, and P.B. Hackett. Regulation of early expression of transgenes in developing fish. *Transgen. Res.*(Submitted), 1992.
24. Moav, B., Z. Liu, Y. Groll and P.B. Hackett. Selection of promoters for transgenic fish. *Mol. Marine Biol. BioTech.* 1: 338-345, 1992.
25. Palmiter, R.D., R.D. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.E.. Birnberg, and R.M. Evans. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300: 611-615, 1982.

26. Shears, M.A., G.L. Fletcher, C.L. Hew, S. Gauthier and P.L. Davies. Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). *Mol. Mar. Biol. Biotech.* 1: 58-63, 1991.
27. van der Krol, A.R., J.N.M. Mol and A.R. Stuitje. Modulation of eukaryotic gene expression by complementary RNA or DNA sequences. *BioTechniques* 6: 958-975, 1988.
28. Xu, L., D.V. Mourich, H.M. Engelking, S. Ristow, J. Arnzen, and J.C. Leong. Epitope mapping and characterization of the infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesized in *Escherichia coli*. *J. Virol.* 65: 1611-1615, 1991.
29. Zhang, P., M. Hayat, C. Joyce, L.I. Gonzalez-Villasenor, C.M. Lin, R.A. Dunham, T.T. Chen and D.A. Powers. Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio* (Linnaeus). *Mol. Rep. Dev.* 25: 3-13, 1990.
30. Zhu, Z., Hm Xu, G. Li, Y. Xie and L. He. Biological effects of human growth hormone gene microinjected into the fertilized eggs of loach *Misgurnus anguillicaudatus* (Cantor). *Kexue Tongbao* 31: 988-990, 1986.

APPENDIX II

LCMR Final Status Report - Summary - Research

July 1, 1993

LCMR Final Status Report - Summary - Research

I. Aquaculture Facility Purchase and Development and Transgenic Gamefish Growth Studies - Fisheries 24

Program manager:
Ira R. Adelman
Department of Fisheries and Wildlife
College of Natural Resources
University of Minnesota
1980 Folwell Avenue
St. Paul, MN 55108
612-624-3600

A. M.L. 91 Ch __ Sec. __ Subd: 8 (b) Appropriation: $1,200,000
Balance: $0

Aquaculture Facility Purchase and Development and Genetic Gamefish Growth Studies: This appropriation is to the University of Minnesota, College of Natural Resources, to acquire and develop an aquaculture facility and to continue research on genetically engineered gamefish.

B. Compatible Data: NA

C. Match Requirement: $364,400 from the University of Minnesota

II. Narrative

Aquaculture is a rapidly growing activity in Minnesota with excellent potential for future expansion as a significant commercial industry. Although faculty at the University of Minnesota have played a leading role in the development of aquaculture in the mid-west through research, extension, and classroom education, efforts to conduct research and extension/demonstration projects are limited by lack of adequate facilities.

Over the past 3 years, the Minnesota Transgenic Fish Group has inserted genes conferring growth enhancement into Minnesota gamefish (trout, walleye, northern pike, salmon). Extensive analysis must continue on these fish and their offspring, the largest group of transgenic fish in North America, testing for level of expression of the genes, their growth effects, and stable transmission to succeeding generations. New strains of transgenic fish are to be developed seeking more optimal expression and growth.

This proposal provides a means of providing adequate facilities for aquaculture/fisheries research and demonstration and for holding genetically engineered fish and continues analysis and improvement of genetically engineered fish.

III. Objectives

A. Construct an aquaculture/fisheries research and demonstration facility on St. Paul Campus of the University of Minnesota.

A.1. Narrative: Construction of this facility will enable University faculty to conduct applied research, demonstration projects, and workshops and will provide space to hold the genetically engineered fish from Objective B of this project as well as from previously funded LCMR projects.

A.2. Procedures: Preliminary plans and estimates have been developed by University staff and a consultant will be hired to confirm the final design. In order to conserve water and energy the facility will be operated largely as a closed system facility. Blowdown water will be discharged to the storm sewer after first passing through a killing field to ensure the destruction of living organisms. Water will most likely be obtained from two existing 800 foot deep wells that were drilled for the now terminated Aquathermal project, previously funded by LCMR. Design and construction contracts will be awarded following appropriate University bidding and contracting procedures.

A.3. Budget

	LCMR Funds
a. Amount Budgeted:	$1,004,000
b. Expenses:	$1,004,000
c. Balance:	$0

A.4. Timeline for Products/Tasks

	Jul91	Jan92	Jun92	Jan93	~~Jun~~ Dec93
Marine appraisals	********				
St. Paul Campus Planning		******			
Consultant hiring			***		
Program development			****		
Design				******	
Construction					**********
Final report					*****

A.5. Status: WORK PROGRAM AMENDMENT
After approval by the Commission of the work plan change in order to build the facility on St. Paul Campus, a consultant was hired to develop the design. The consultant, J.M. Montgomery

Engineering in association with Lambert and Beck Architects, along with the University planning committee completed program planning in late July. As part of the planning evaluation we compared the cost of putting the facility in a new building versus remodeling a portion of the Agricultural Engineering Shops building. The latter proved to be more cost effective, and thus the facility will be located in the existing building. From late July until late October, the consultant and the University negotiated the final contract terms. The consultant and the planning committee finalized the design in late October. Because of numerous unavoidable delays (see strike throughs below) the LCMR approved an extension of the completion date to December 31, 1993. ~~construction drawings will be completed by mid-December, and the bidding process to hire a contractor for the construction will begin in early January. A few of the major equipment purchases are now out on bids.~~ The first set of blueprints were submitted by the consultant on January 14, 1993, somewhat behind schedule, and they were deemed totally inadequate by U. of M. Facilities Management. Review comments (31 pages) were returned to the consultant on February 10. The second set of blueprints were received on March 3, slightly improved, but still inadequate. Review comments (26 pages) were returned on March 29. The third set of blueprints were received on April 22 and these were good except for the need to redesign a door which delayed the approval by the University for 2 weeks until June 3. Advertisement for a construction contractor appeared in the Construction Bulletin on June 11 and 18 and bids will be opened on July 1. With the approval of the blueprints the funds have been encumbered for construction but not liquidated. The contract requires 150 days for completion, so if all goes well, the facility will be finished by mid-December 1993. The $44,000 that was transferred from Objective B to A after approval of the LCMR has been encumbered for the purchase of tanks. Bids from potential vendors will be opened on June 25.

~~According to University staff and J.M. Montgomery, Consulting Engineers Inc., the project should be completed sometime in September 1993. Knowing that unforeseen delays frequently occur during major construction projects, the project completion date has been extended to December 31, 1993. The reasons for the no cost extension are as follows:~~

~~1) The first 7 months of the project were spent evaluating the purchase of the Marine Minnow Farm and concluding that it was not a viable alternative. The current work plan, to build on St. Paul Campus, was not undertaken until after the Commission approved the work plan change on February 6, 1993. Most of the approximately $10,000 that have been spent from the budget thus far were for appraisal fees and water analysis related to the Marine Minnow Farm evaluation.~~

~~2) Because of state rules for bidding and the need to go through the State Designer Selection Board, a consultant was not hired until after May 11, 1993. Everyone cooperated to the maximum to keep this process on the fast track, but rules for timing of the bidding process and the normal difficulty in scheduling meetings among numerous participants prevented any quicker action.~~

~~3) There have been a number of delays in the University's negotiating the contracts with J.M. Montgomery which were completely out of the control of the project manager. According to University staff, the consultant was very slow to respond to proposals made by the University.~~

~~4) Now that the contract with the consultant current work plan is underway, the realistic time it will take for design completion, letting a contract for bids, hiring the contractor, purchasing equipment, and actual construction is likely to be a little less than a year. LCMR funds should all be encumbered by July 1993.~~

~~Since the final product will be a functioning facility, the LCMR will be able to clearly demonstrate the results of their expenditure.~~

A.6. Benefits: Construction of the facility will provide a state of the art facility for applied aquaculture/fisheries research, demonstrations, and workshops and will provide adequate space for holding the genetically engineered fish. Faculty at the University will be able to take advantage of funding sources for these kinds of projects from such organizations as the North Central Regional Aquaculture Center, Greater Minnesota Corporation, USDA, Minnesota DNR and the Fish and Wildlife Service. The previous investment in research of approximately $1,000,000 to develop the existing group of transgenic fish will be preserved.

B. **Continued analysis (growth, performance, molecular, and breeding) of transgenic fish; ~~creation of new transgenic fish to test new genes.~~**

B.1. Narrative: Extensive analysis must continue on the current transgenic fish, which are a resource representing approximately $1,000,000 in research funding. Analysis of growth potential, transgene effect, and inheritance of the transgene is required. Continuation of effort is needed to isolate other growth promoting fish genes and their controlling elements, with subsequent transfer into fish, establishing new transgenic fish lines. Transgenic fish will not be released into the wild in the foreseeable future, however, ecological studies addressing this important issue are presently in the planning stage.

B.2. Procedures: **DNA analysis.** The presence and physical state of transgene DNA within existing transgenic fish is characterized by Southern blot hybridization analysis. Isolated fin DNA from juvenile fish (Hallerman et al. 1990: by sodium dodecyl sulfate lysis (SDS) and proteinase K digestion) is digested with excess BamHI restriction endonuclease, electophoresed through a 0.8% agarose gel, transferred onto nylon membranes by Southern blotting and then probed for the presence of transgenic DNA. Probes used to identify positive fish consist of either pRSV/bGH, pRSB/npGH or FV-2/csGH labeled with ^{32}P by the random priming method.
RNA analysis. The presence of transgene poly (A)+ RNA (Glisin, et al. 1978) will be analyzed by dot-blot hybridization employing radioactive probes labeled to high specific activities (ca. 5 x 10^8cpm/ug) to determine whether any quantitative differences exist between various cell and tissue types in a given transgenic fish, and by Northern blot hybridization (Thomas, 1980) to determine if any qualitative differences can be detected. In situ hybridization

will be used to analyze both whole sections of embryos and cross sections of juvenile fish to identify the stage in embryogenesis as well as the specific tissues type in which transgene related sequences are being expressed (Haase et al. 1984). This technique offers several advantages over the standard methods of hybridization including: 1) very small tissues samples can be used directly without RNA extraction and purification; 2) the sensitivity of this technique will allow the detection of a minimal amount of transcripts from just a few cells in a tissue preparation (an extremely important issue for mosaic founder fish); and 3) most importantly, expression can be localized in tissues thereby providing information about the specific types of cells within a tissue sample that are expressing specific transgenic sequences. Briefly, sections from formaldehyde-fixed and paraffin-embedded tissues are attached with the aid of an adhesive to microscope slides that have been acid washed, treated with Denhardt's solutions and the acetylated. Following deparaffination with xylene and dehydration, sections are acid extracted, neutralized, and treated sequentially with digitonen, proteinase K, and DNase. Large samples can be serially examined while frozen in carboxy methyl cellulose by sectioning with a cryostat. Tritium-labeled DNA probes are hybridized for two days at room temperature in formamide-destran sulfate solutions. Following extensive washing, slides are covered with photographic emulsion and developed at appropriate times. Positive hybridization is selected by either light of electron microscopy following hematoxylin staining as evidenced by an excess of silver grains over cells as compared to control samples.

Growth hormone radioimmunoassay. Blood scrum samples collected from dorsal aortae of two month old fish will be analyzed for the presence of transgene GH protein by radioimmunoassay. Because the growth hormone containing constructs include the signal peptide sequence (responsible for secretion of the hormone into the blood stream under normal circumstances), detection of transgene GH protein is expected. Radioiodinated transgene GTH is prepared by adding four iodo-Beads (Pierce Chemical Co., Rockford, IL) to a vial containing 1.5 mCi ^{125}I and 0.2 ml buffer (0.25 M Na_3PO_4 pH 7.5). After 5 minutes, 14 mg bovine growth hormone (USDA) solubilized in 0.01 M. $NaHCO_3$ diluted with 20 ml of buffer is added and incubated for 15 min. The mixture is purified by column chromatography using Bio-gel P-30 (Bio-Rad) equilibrated and eluted with 0.1% bovine serum albumin (BSA)-barbitol buffer pH (Sigma) as three peaks. The middle peak is diluted in 1% BAS-0101 phosphate-buffered saline M. (PBS) pH 7.5. Anti-serum to ovine GH (NIAMDD-anti-oGH) is diluted 1:5,000 in 1% normal rabbit serum-.05 M EDTA-PBS pH 7.0, and 0.1 ml dispensed into assay tube containing 0.3 ml 1% egg albumin-PBS pH 7.0 and 0.32 ml test plasma. After 24 hours later by the addition of 0.1 ml precipitating antiserum (sheep anti-rabbit gamma globulin) and incubation for 48 hours. Samples are centrifuged at 1,500 X g for 20 min and counted in a Beckman gamma counter. Assay sensitivity is approximately 1 ng/tube or approximately 1 ng/ml (Wheaton et al., 1986).

Immunohistochemical detection. Fin tissue collected from individual fish in the microinjected groups can be fixed, sectioned, and mounted on slides using standard clinical techniques. Slides are deparaffinized by a 30 min room temperature dip in xylol, followed by two 5 minute dips in 100%, 90%, and 70% ethanol. Slides are washed three times in PBS pH 7.6 for ten minutes and in PBS for five minutes. Blocking is by incubation in a 1X dilution of normal sheep serum for 30 minutes at 37°C, followed by a gentle wiping. Slides are incubated in polyclonal rabbit anti-bGH diluted 1:100 in normal sheep serum overnight at 4°C, washed three times in PBS for 10 minutes, and incubated with goat anti-rabbit Ig diluted 1:2,000 in normal sheep serum for 30 min at room temperature. Slides are washed three times in PBS for 10 min, and incubated with the DAB (Diaminobenzidine tetrahydrochloride) substrate (5mg DAB in 20 ml 0.03% H_2O_2 in PBS) for 10 min at room temperature in the dark. Slides are washed three times in PBS, counterstained in 0.15% methylene blue for 10 min, washed twice in tap water, dehydrated by a series of dips in 70%, 95%, and 100% ethanol, dipped in xylol twice, and mounted with Permount. growth of selected fish is monitored monthly for weight and length grain.

B.3. Budget

	LCMR Funds
a. Amount Budgeted:	$196,000
b. Expenses	$170,034
b. Balance:	$0

B.4. Timeline for Products/Tasks

	Jul91	Jan92	Jun92	Jan93	Jun93
Analyze existing transgenic fish	**********				
Breed transgenic fish at maturity	**********	**********	**********		
Analyze offspring			**********	**********	
Growth studies of offspring			**********	**********	
Analyze breeding data				**********	*****
Designate broodstock					*****

B.5. Status: WORK PROGRAM AMENDMENT

1. Current Status of Transgenic Fish

a) Northern Pike:

1988 Founder stock (RSVbGH): 36 founder fish remain from the 1988 founder stock (7 males and 29 females), including all five previously identified as positive for the transgenic Growth Hormone (GH) gene construct. 24 control siblings are also alive. Growth studies have indicated an enhancement effect of the extra growth hormone gene in some cases. One two-year old (1990) cross exists as of 6/93, however only 2/25 remaining siblings contain an extra growth hormone gene (other positive siblings died). These positive fish have not demonstrated enhanced growth, however rearing conditions have been much less than ideal. In 1991, 3/8 crosses of the 1988 stock were positive for the transgenic GH gene by Southern blot analysis. Only 12 fish remain from these crosses. Many of the remaining

1988 founder fish spawned during the month of May 1993: 9 males were mated with 15 females to yield 23 crosses (2 of these are control sibling crosses). As of 6/93, 18 crosses have had various degrees of success hatching. These crosses will be screened for transmittance of transgene in ~June/July, 1993.

1989 Founder stock: In April 1991 due to limited space on the St. Paul campus, these fish were split and moved to hatcheries in Cohasset and New London, MN. The Cohasset fish all died from a facility malfunction. The New London fish went through strong negative selection due to health problems (well water on the St. Paul campus is from an aquifer and thus somewhat sterile; whereas the NL water was taken from the bottom of a outdoor pond and thus contains all of the parisites a fish in the wild may succumb to, winter water temperatures was only 39`F). Only the fittest fish survived, which somewhat surprisely did not include the known transgenic founder fish. Only 54 of the ~200 fish (>50% known to be transgenic) that were moved to NL returned to the St. Paul Campus, and only 2 of these were known to carry transgenic GH gene from blood and tissue assays by Southern blotting. As of 6/93 only 33 fish survive, with only one of the known transgenic founders being among them. However, this female is the fourth largest amongst its siblings. The other 32 are potential mosaics; they are not positive in fin and blood samples but may be positive in their gonads. Consequently, these fish are being kept until gametes can be obtained and screened for transgenic GH gene. The remaining 1989 founders did not produce gametes in 1993.

b) Rainbow trout:

1989 Founder stock with the bGH gene: No founders have survived. 25 crosses were made in 1990. 6 of 14 crosses tested at the embryo stage were found, by Southern blotting of DNA from selected samples of embryos, to be positive for extra bGH genes. However, when checked again after survival to the fingerling stage, only two crosses still had positive offspring, as of 6/93 there are 45 siblings alive of which only 3 possess transgenes. There is no apparent growth enhancement in these fish.

1990 Founder stock with the csGH gene: The health of these fish deteriorated as the 1993 spawning season approached. 39 fish died during this stressful period. The reason for these deaths is not understood, but it also affected gamete quality in the remaining living fish. Only 4 females gave eggs of sufficient quality to be fertilized during Febuary and March of 1993. Different males were used to ferlize these eggs to yield 9 crosses. 3 of these hatched and are presently being screened for transgene transmittance. Two of these crosses have ~1000 siblings each, the third cross only has 26 siblings. Of the remaining 61 founder stock, 12 fish are still alive that are known to be positive for extra csGH genes. Fortunately, all of the fish known to transmit the extra csGH genes are still alive. The other founders are all potentially mosiac and thus are potentially capable of transgene transmittance (when these fish eventually breed we will be able to determine if transmittance is possible). As mentioned in previous updates, all control founder siblings (mock injected, non-transgenic fish) were sacrificed due to space limitations thus destroying any ability to determine growth enhancement in the founder stock. There are three positive crosses from the 1992 mating season that were screened for transgene transmittance (~400 fish) in May 1993. Only one of these appears to have any positive offspring remaining; frequency ~1% for transmittance.

c) Atlantic salmon:

1990 Founder stock with the csGH gene: As of 6/93, 118 founder fish are surviving; 5 of these are known to be positive for transgenic csGH gene within their fin tissue. The remaining fish are potentially mosiac, and thus have a chance of having transgenic gametes. 20 early-maturing female fish were bred in late 1992; 21 crosses were made of which 17 were successfully fertilized. Unfortunately, most of the embryos died shortly before hatching. There are ~130 fish remaining alive from 8 of these crosses. The low survivability might be due to the early maturation of the gametes, a full year early. Whether the transgene was passed on in these crosses is unknown as of yet. 25 males produced gametes, however, analysis of the sperm DNA showed only 1 possessed the transgene. The growth performance of the atlantic salmon founder fish is impossible to evaluate since all control siblings have been sacrificed due to space limitations. Nearly all founders should spawn in 1994, and the gametes are expected to be of high quality.

d) Walleye:

1990 Founder stock with the csGH gene: All have died. No progeny were obtained before death..

e) Summary:

Although the number of surviving founder fish is low, many are apparently transgenic. Only one or two actively expressing fish are required to serve as broodstock for future generations. The outlook positive.

2. Current Status of Transgenic Fish Culture

Owing to the requirements that the transgenic fish be maintained indoors, we have had to move the fish to several hatcheries throughout the state of Minnesota. These moves, accompanied with occasional periods of poisoning with chlorinated water and high/low temperature water, severely stressed the potentially transgenic fish. Consequently, with financing obtained from the University of Minnesota/Legislative Commission of Minnesota's Resources, a new facility is being constructed that will house the products of

this project. This should i) eliminate the problems we have encountered in maintaining adult fish to reproductive age, and ii) provide adequate space to determine the enhanced growth of second generation fish.

We continually breed the transgenic fish but currently have to discard older fish to continue new lines. If the new facility is constructed soon enough, we will be able to raise the offspring of atlantic salmon mated in December 1992, the rainbow trout offspring from March 1993 matings, and the northern pike offspring from May 1993 matings. This would provide us with a large number of potential broodstock for behavioral and performance analysis of transgenic fish by Prof. Kapuscinski.

3. General observations on transgenic fish.

Although these studies were not designed to evaluate the overall fitness of fish transgenic for growth hormone genes, preliminary evidence strongly suggess that their fitness is low. Several lines of evidence supports this:

i) Mendelian inheritance of transgenes would be expected to be ~>/= 50%, however when hatchlings have been screened we have yet to see more than 20% transmittance (usually ~5-10%). This suggests that selection is occuring during embryo maturation that preferentially effects the transgene carrying siblings.

ii) in every documented case to date, once a positive cross has been identified (shortly after hatching), by the time these fish are one year of age most of the siblings possessing the transgene have died. Again suggesting a low fitness value for those possessing transgenes.

iii) perhaps the most convincing evidence is the lack of survival by transgenic fish moved to the New London facility. These fish went from the near sterile environment at the St. Paul Campus to an environment filled with every normal pathogen a wild fish might encounter (water was taken from the bottom of a outdoor pond). Of the 200 fish that were moved there, 49 were known to be transgenic. Only 2/54 returning fish were carriers. Today only one of positive fish survive suggesting that their over all fitness did not match their nontransgenic co-siblings (some of the survivers might be mosiac though).

This evidence might suggest that other less pleiotrophic growth enhancing genes should be used in place of GH genes.

~~4. Current Status of Gene Cloning.~~

~~We do not have facilities to house new transgenic fish. When this proposal was submitted, we anticipated that as we made transgenic fish, we would either 1) move them to outstate hatcheries for breeding or 2) release the fish into outside facilities used by the MN DNR for evaluation. However, we have found that movement of transgenic fish from one facility to another in the past three years induces considerable stress to these animals and this stress was a major contributor to the loss of nearly 80% of our transgenic stocks. Moreover, in the past year the state has re-examined the issue of release of genetically engineered organisms and EQB has adopted rules which are more stringent than anticipated. In order to put our transgenic fish into outdoor facilities, we will need to obtain more information on their feeding and behavior. These experiments are currently being designed. In the meantime, the fish must remain indoors. Our abilities to keep fish has been curtailed because as the fish increase in size, the number per tank must be lowered. As a result, we are constantly culling fish that are not transgenic. Without keeping non-transgenic fish (controls), we cannot do growth studies.~~

~~Consequently, we cannot keep the fish we have alive and at the same time create new lines of fish. We have the vectors and the genes, and through federal funding obtained on the basis of the LCMR results to date, we are continuing to develop new growth enhancing genetic constructs. In particular, a U.S. Department of Agriculture grant is funding investigations into alternative growth stimulating genes, like the c-ski gene which is specific for muscle tissue and therefore lacks some of the unsettling aspects of more global hormones like growth hormone. But, we need expanded facilities for these fish and until we have them, we cannot produce more fish.~~

~~Accordingly, we request that the $44,000 budgeted for the development of new lines of transgenic fish be used instead to purchase tanks to hold both the growing offspring of our current transgenic fish and the fish we want to produce but have no room for at present. The cost for construction of the new facility (Objective A) has left no money for the purchase of large tanks for transgenic fish, but there is space for the tanks. The fish we proposed to create will be produced, using the money from the USDA grant ($250,000), mentioned in the previous paragraph, and both the old and the new fish will be maintained in the new facility if the tanks become available. Thus, we think it best in terms of the goals of the LCMR program to redirect the funding originally targeted for more transgenic fish to the purchase of tanks to maintain the valuable fish we already have and those we planned to make.~~

B.6. Benefits:

a. Determination of economic potential of some 2000 existing transgenic fish (walleye, northern pike, salmon, rainbow trout);
b. Develop broodstock of optimal transgenic fish for research and possible commercial application;
c. Establish breeding protocols for maximal analysis of future transgenic fish;
d. Possible finer control over growth enhancement through use of other growth promoting genes [e.g. insulin-like growth factor (IGF-1), growth hormone releasing factor (GHRF)];
e. Isolation of growth promoting genes for possible expression and use of product as feed supplement;
f. Creation of new lines of transgenic fish to complement growth hormone lines in existence.

IV. Evaluation:

For the FY 92-93 biennium the program can be evaluated by: 1) successful construction of a state of the art aquaculture/fisheries research and demonstration facility 2) identification and spawning of the transgenic fish showing the best growth enhancement potential, 3) characterization of the status of the transgene in expressing fish, and 4) successful injection of new constructs containing fish growth hormone genes in the appropriate species. Long term evaluation of the project's success will be determined by future success in conducting applied research, demonstrations, and workshops at the facility to benefit the development of aquaculture in Minnesota and in developing broodstocks of transgenic fish with enhanced growth characteristics.

V. Context:

A. Objective A---The existing laboratory on St. Paul Campus is lacking both water flow capacity and space. When that laboratory was completed in 1969, research was largely confined to working with small species or young fish, mostly in pollution related studies. In recent years, emphasis in research and teaching has shifted to large species in sports fishing or aquaculture situations.

The rapid growth of interest in aquaculture has provided new opportunities for research and extension that cannot be pursued in the existing facility. The recently established North Central Regional Aquaculture Center has provided a new source of funds for aquaculture research and technology transfer. Although University faculty are participating and even leading the way in much of the Center's activities, only a small portion of the Center's funding has been awarded to the University because facilities are inadequate. Approximately $1.35 million in total research funding has been lost over the last 3 years as a result of not having adequate facilities.

In comparison to Minnesota, most state universities in this region have much more extensive facilities, generally built or upgraded in the last 5 years. Although some of the research done at these locations will be applicable to Minnesota, the more local concerns will not receive adequate attention, and Minnesota will fall behind in its attempt to develop an aquaculture industry.

Objective B---Over the past 4 years, the Minnesota Transgenic Fish Group has established itself as one of the most active fish genetic engineering groups in the world and has initiated a large effort in the economic improvement of fish via genetic engineering, with primary focus on the introduction of extra growth promoting genes by gene transfer techniques. Currently, about 3000 transgenic fish are being maintained on the University campus (Hodson Hall) and off campus (DNR, MN Power). These fish, of four species (northern pike, walleye, salmon, and rainbow trout), are being cultured to determine the efficiency of the transfer and growth enhancement, to study the biology of such transgenic organisms, and to mature for breeding to determine inheritance of the trait and to establish strains of these species with improved growth rates. This latter goal necessitates maintenance of the fish for 2-4 years per generation. To date, transgenic fish have been produced using a bovine growth hormone gene and viral promoters into salmon, pike, trout, and walleye, and by inserting a salmon growth hormone gene into salmon and pike. Our ideal transgenic fish, however, contains only pieces of added DNA that originally came from that species, i.e. an added northern pike gene into northern pike. The proper transfer system and vector has been produced by our group, and the northern pike and walleye growth hormone genes have been isolated and cloned. This step is necessary to meet regulatory concerns for human consumption of these fish and to establish the proper strain of recombinant fish for ecological studies.

Thus current work has produced a large number of transgenic fish which must be further analyzed to determine the effectiveness of the transferred gene and its passage frequency to future generations. The production of the ideal transgenic fish has been begun by inserting the salmon gene, however these fish also must be analyzed and bred, requiring culture until sexual maturity. The production of transgenic pike and walleye using the species specific gene needs to be begun, and all components (vector, methodology, and cloned genes) are in place. This will enable the production of optimally useful transgenic fish for commercialization when current questions concerning dangers of genetic-engineered fish are resolved.

B. Objective A---Construction of the aquaculture/fisheries research and demonstration facility will provide the kind of facility needed to advance aquaculture in Minnesota. Construction of this facility will provide a state-of-the-art aquaculture/fisheries research and demonstration site.

Objective B---The proposed work will add to existing data and work in progress by enabling the complete analysis of existing fish, allowing the maturation and breeding of the existing fish, and by producing better transgenic fish (northern pike and walleye) using existing methods and genes. This supplementary work will enable the better selection of transgenic fish for broodstock generation, by analysis of the levels of expression of the transgene in existing fish, and examination of the stability and state of the transgene DNA in the fish. Since each individual fish in the injected population takes up the transgene a little differently, a search of the whole population for the best uptake and function of the gene is necessary. This in turn gives data enabling better success in the future. The proposed work also entails creation of two new populations of transgenic fish, northern pike with an added northern pike growth hormone gene and walleye with added walleye growth hormone genes, both to verify the data gained in these species using a cow growth gene and to establish strains of transgenic fish suitable for commercial development.

C. Objective A---The Department of Fisheries and Wildlife, University of Minnesota has had a long history of conducting pioneering and internationally recognized research in aquaculture and fisheries including two aquaculture projects funded by LCMR (Genetic Engineering of Minnesota Fishes and Improvement of Pond Aquaculture in Minnesota). It is likely that there will be future proposals to LCMR to conduct specific research or demonstration projects at the new facility.

Objective B---The Transgenic Fish Project was begun under Sea Grant (Federal) funding which enabled the initial development of transfer techniques. Other non-LCMR funds (Blandin Foundation

and Greater Minnesota Corporation) have supported much of the work with salmon and trout, creating transgenic rainbow trout with a bovine growth hormone gene that are now just old enough to be bred. Indeed one of the precocious male transgenic trout has shown the gene to be passed on to the next generation. Sea Grant funding has continued to members of the group and has allowed investigation to begin on other growth promoting genes and the possibility of transfer of disease resistance. Sea Grant funding has also aided in the construction of the all-fish expression vector which has been and will be used in producing ideal transgenic strains. Non-LCMR funds have totaled about $820,000 over the past 6 years. The investment has spawned: 1) U.S. Department of Agriculture funding of investigations into alternative growth stimulating genes ($250,000), 2) National Institute of Health funding of alternative model fish systems for testing hormone action, *e.g.*, thyroid hormone receptor activity in zebrafish ($450,000), and Sea Grant funding for additional research on transfer of the growth hormone gene ($120,000).

The LCMR funded the development of the transgenic process for northern pike and walleye and the start of the transgenic population of these fish containing the bovine growth hormone genes. The LCMR money also enabled the cloning of the northern pike and walleye growth hormone genes, and aided in the construction of the expression vector to be used with them. The LCMR funds (1987-89) were critical to getting the project well established and have permitted it to be developed into one of the premier such efforts in the world.

Potential future LCMR proposals may be considered to examine the ecological interaction of wild and transgenic growth-enhanced fish and to extend the technology to solve other genetic-related problems (disease resistance, cold or heat tolerance, etc.).

D. Not applicable.

E. Biennial Budget System Program Title and Budget: Not Available at this Time

VI. Qualifications

1. Program Manager:
   Dr. Ira R. Adelman
   Professor and Head
   Department of Fisheries and Wildlife, University of Minnesota

   Ph.D. Fisheries, University of Minnesota, 1969

   Dr. Adelman has conducted research on the environmental physiology of fishes for over 20 years. Much of that research has been applicable to aquaculture. He is on the research committee of the North Central Regional Aquaculture Center and on the Minnesota Aquaculture Commission. As head of the Department of Fisheries and Wildlife he has been responsible for administration of department programs and a budget as high as $3 million annually including external grants. Dr. Adelman's primary role will be as program coordinator and to oversee work conducted under Objective A.

2. Major Cooperators:

   A. Dr. Anne R. Kapuscinski
      Associate Professor and Extension Specialist
      Department of Fisheries and Wildlife and Minnesota Sea Grant College, University of Minnesota

      Ph.D. Fisheries/Genetics, Oregon State University, 1984
      M.S. Fisheries/Aquaculture, Oregon State University, 1980

      Dr. Kapuscinski has conducted extensive research in aquaculture and fish genetics and has been responsible for an extension program aimed toward the development of aquaculture in Minnesota. In working with farmers and regulatory agencies, she is familiar with the problems associated with effluents from aquaculture facilities and in control systems. Dr. Kapuscinski will be responsible for assisting Dr. Adelman in the completion of Objective A.

   B. Dr. Anthony Faras
      Professor
      Department of Microbiology, University of Minnesota

      Ph.D. Pathology/Virology, University of Colorado Medical Center, 1970

      Dr. Faras's primary expertise is in the areas of viral molecular biology and analysis and the molecular cloning of genes. While most of his work has been in human systems, over the past few years, he has expanded into other animal systems with a special emphasis on fish. He has been a member of the Minnesota Transgenic Fish Group since its inception and has been responsible for the cloning of walleye and northern pike growth hormone genes for use in the group project. Dr. Faras's primary role will be to continue the cloning efforts and perform molecular analysis on the transgenic fish under Objective B.

   C. Dr. Perry Hackett
      Professor
      Department of Genetics and Cell Biology, University of Minnesota

      Ph.D. Biophysics/Genetics, University of Colorado Medical Center, 1974
      M.S. Biophysics/Genetics, University of Colorado Medical Center, 1970

Dr. Hackett has been a member of the Minnesota Transgenic Fish Group since its inception and has been responsible for the development of an all-fish expression vector for use in transferring genes. He and his group are involved in the genetic manipulations needed to permit optimal expression of the cloned genes in the transgenic fish. Dr. Hackett will continue his vector construction and analysis, seeking to improve the vector for use under Objective B.

3. Scientific Reports Published or in Press since the inception of the grant:

1) Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1991). Importance of the *CArG* box in β-actin gene expression. Gene 108:211-217..

2) Gross, M.L., Kapuscinski, A.R. ,Schneider, J.F., Liu, Z., Moav, N., Moav, B., Myster, S.H., Hew, C., Guise, K.S., Hackett, P.B. and Faras, A.J. (1992). Growth evaluation of northern pike(*Esox lucius*) injected with growth hormone genes. Aquaculture 103: 253-73.

3) Schneider, J.F., S.H. Myster, P.B. Hackett, K.S. Guise, and A.J. Faras (1992). Molecular cloning and sequence analysis of the cDNA for northern pike (*Esox lucius*) growth hormone gene. Mol. Mar. Biol. Biotech. 1: 106-112.

4) He, L., Z. Zhu, A.J. Faras, K.S. Guise, P.B. Hackett, and A.R. Kapuscinski (1992). Characterization of AluI repeats of zebrafish (*Brachydanio rerio*). Mol. Mar. Biol. Biotech. 1: 125-135.

5) Moav, B., Z. Liu, Y. Groll, and P.B. Hackett (1992). Selection of promoters for transgenic fish. Mol. Mar. Biol. Biotech. 1: 338-345.

6) Moav, B., Z. Liu, N.L. Moav, M.L. Gross, A.R. Kapuscinski, A.J. Faras, K. Guise, and P.B. Hackett (1992). Expression of heterologous genes in transgenic fish. IN: *Transgenic Fish* (C.L. Hew and G.L. Fletcher, eds.) World Scientific Pub. Co., pp. 120-141.

7) K. Guise, P.B. Hackett, and A.J. Faras (1992). Transfer of genes encoding neomycin resistance, chloramphenicol acetyl transferase and growth hormone into goldfish and northern pike. IN: *Transgenic Fish* (C.L. Hew and G.L. Fletcher, eds.) World Scientific Pub. Co., pp. 120-141.

8) Hackett, P.B. (1992). The molecular biology of transgenic fish. IN: *Biochemistry and Molecular Biology of Fishes*. (P. Hochachka and T, Mommsen, eds.) in press.

9) Moav, B., Z. Liu, L. Caldovic, A.J. Faras, and P.B. Hackett (1993). Regulation of early expression of transgenes in developing fish. Transgenic Research 1: 153-161.

10) Ivics, Z., Z. Izsvak, and P.B. Hackett (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Mol. Mar. Biol. Biotech. 2:162-173.

11) Caldovic, L., S. Fahrenkrug, J. Breuer, and P.B. Hackett ( ). Piscine expression vectors with polycloning sites. in preparation.

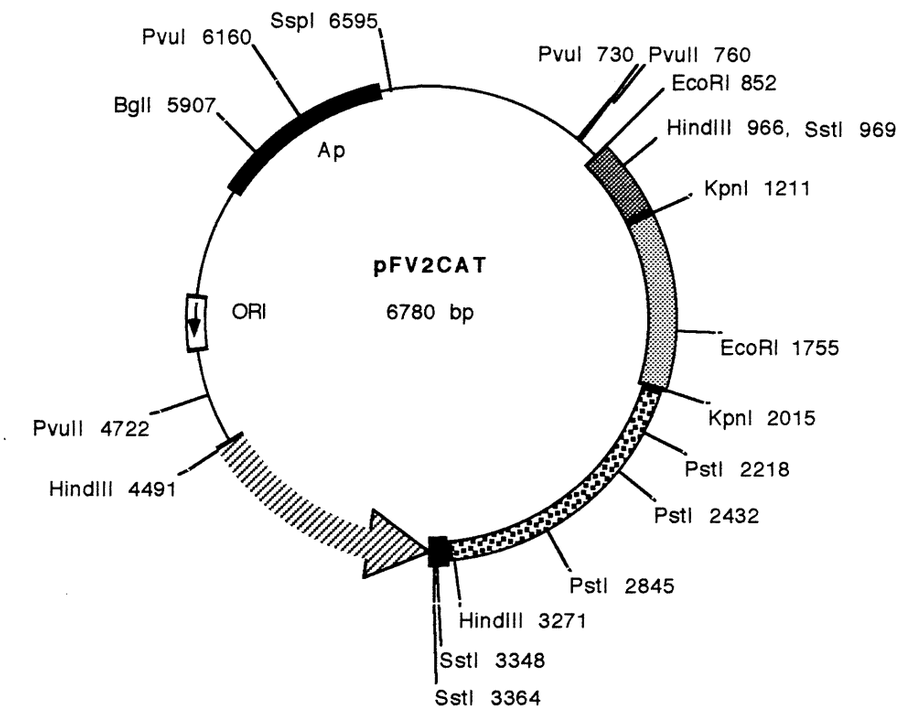
VII. Reporting Requirements

Semiannual status reports will be submitted not later than January 1, 1992, July 1, 1992, January 1, 1993, and a final status report by June 30, 1993 for Objective B and December 31, 1993 for Objective A.

The following pages show the most recently developed vectors and their patterns of expression in microinjected zebrafish (Caldovic et al., manuscript in preparation.)

APPENDIX III

Published Reports



CSGH polyA signal

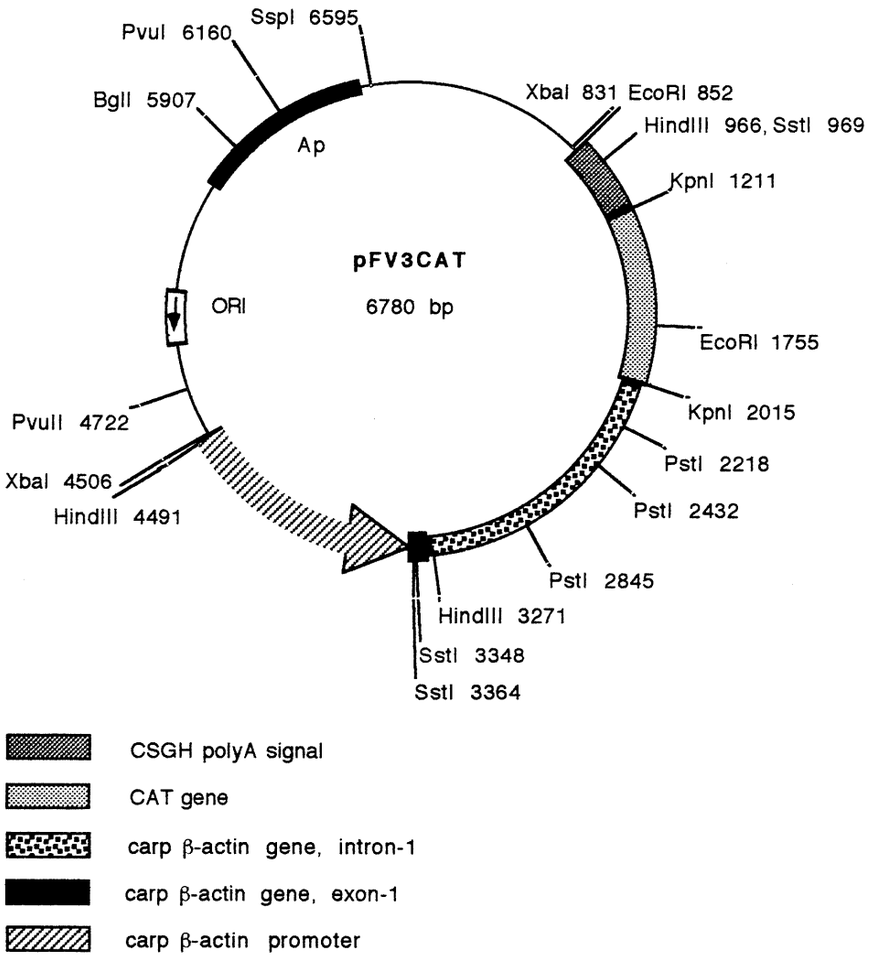
CAT gene

carp β-actin gene, intron-1

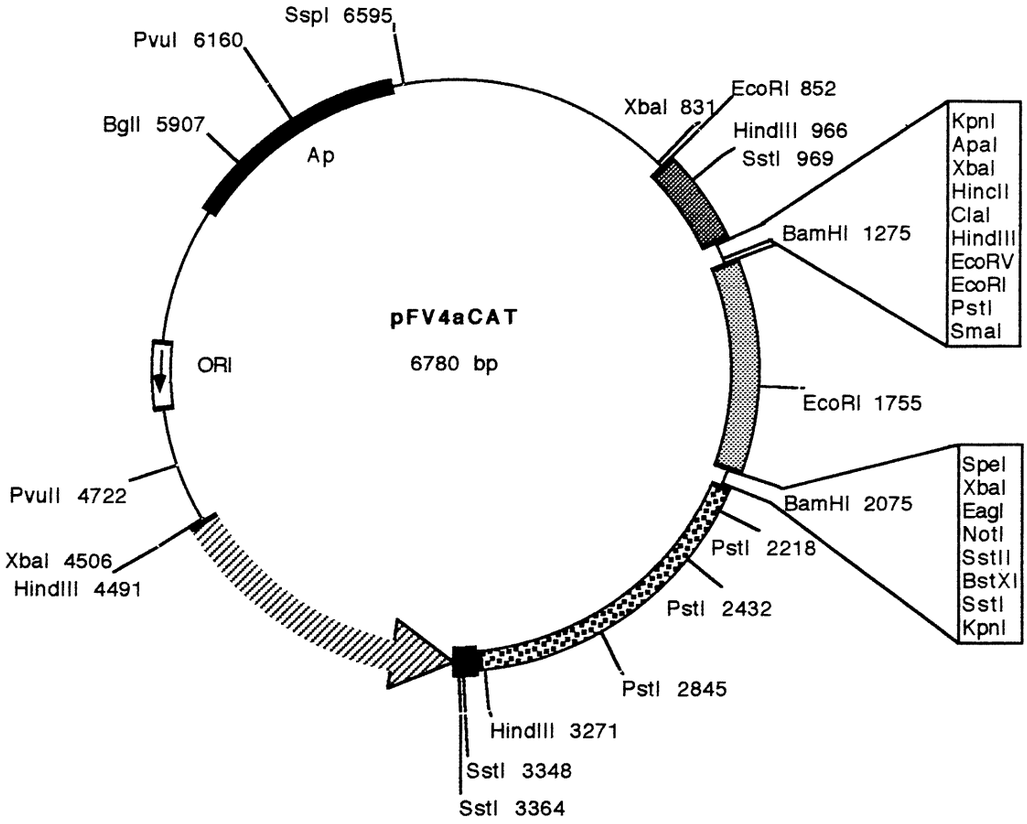
carp β-actin gene, exon-1

carp β-actin promoter

Plasmid name: pFV2CAT
Plasmid size: 6780 bp
Constructed by: Zhanjiang Liu
Construction date: 1989
Comments/References: Biotechniques paper.



Plasmid name: pFV3CAT
Plasmid size: 6780 bp
Constructed by: Zhanjiang Liu
Construction date: 1991
Comments/References: Not published yet. The same expression as in pFV2CAT.



CSGH polyA signal

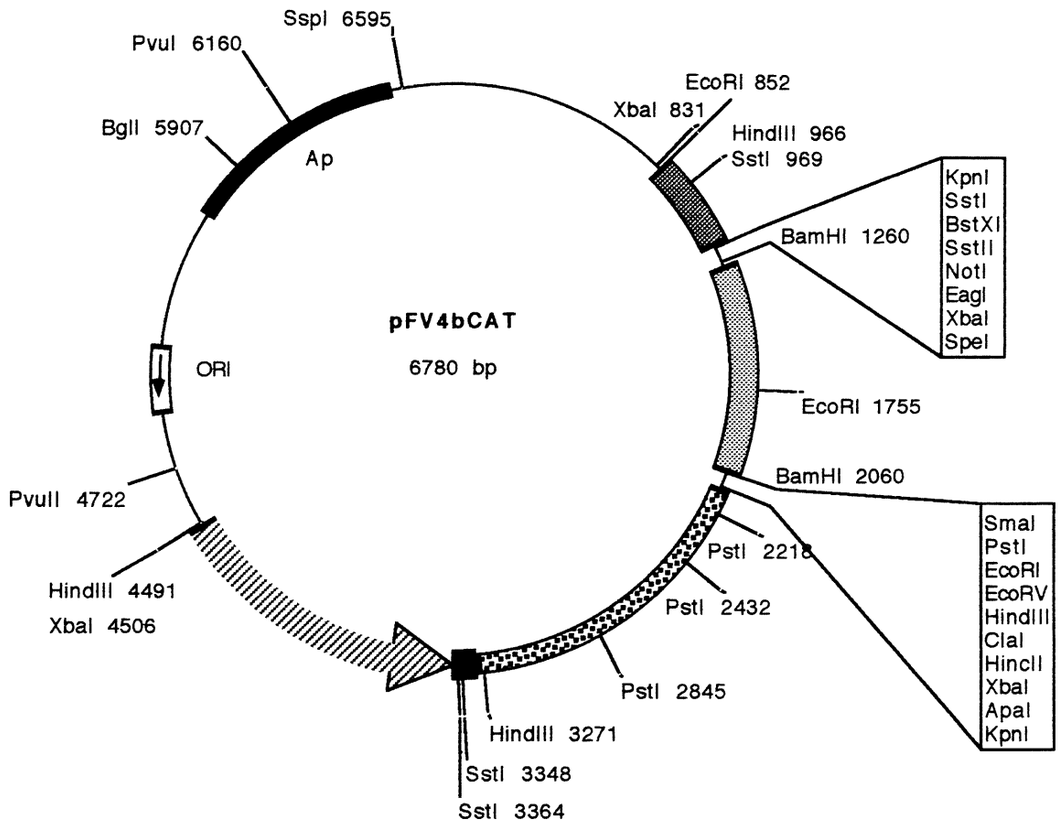
CAT gene

carp β-actin gene, intron-1

carp β-actin gene, exon-1

carp β-actin promoter

Plasmid name: pFV4aCAT
Plasmid size: 6780 bp
Constructed by: Hackett lab
Construction date: 1992
Comments/References: CAT gene is cloned into BamHI site of the polycloning region



CSGH polyA signal

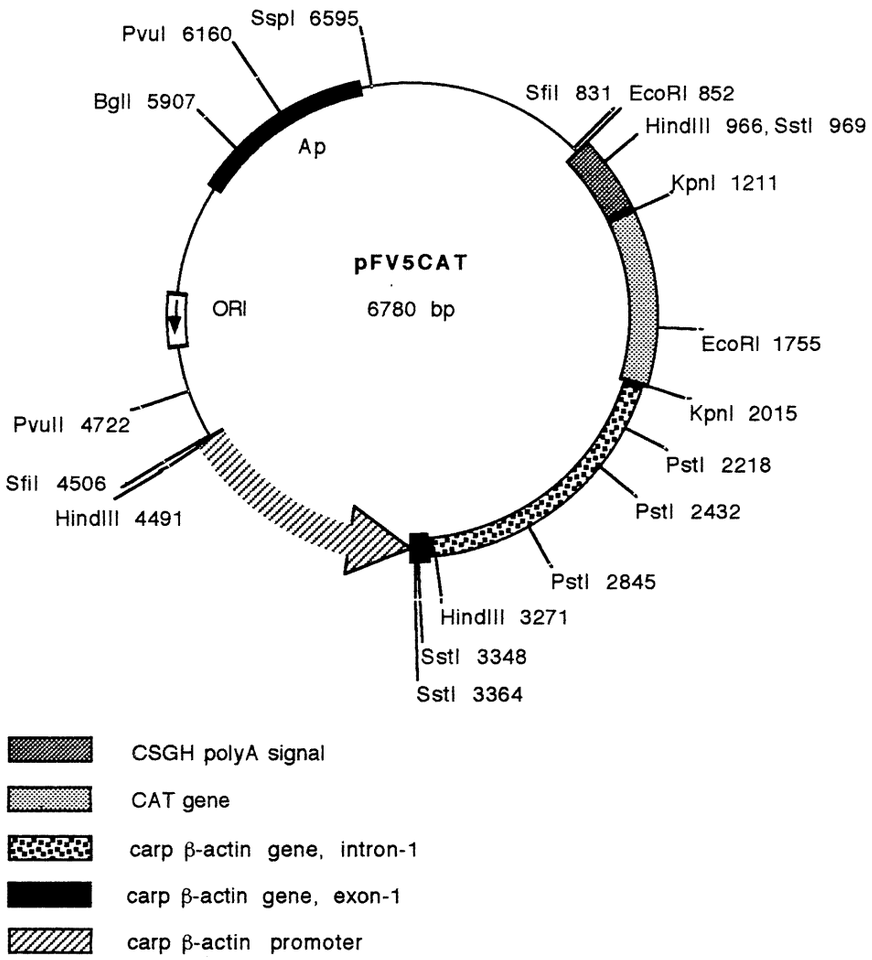
CAT gene

carp β-actin gene, intron-1

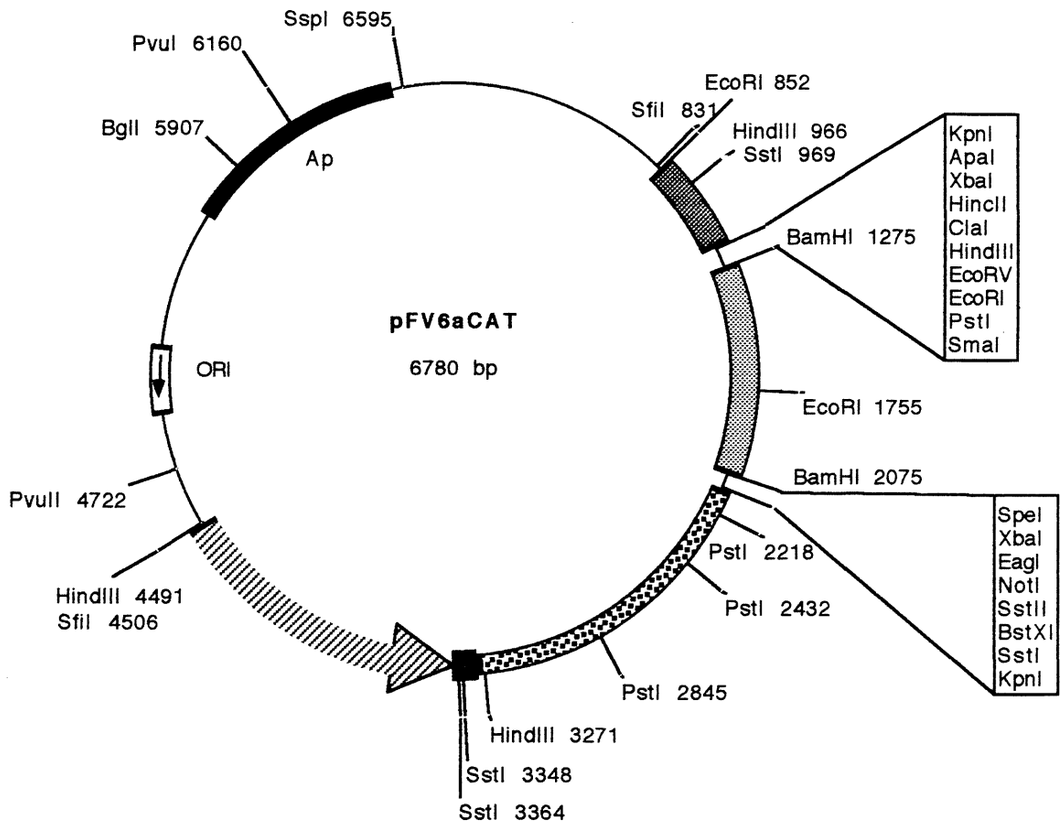
carp β-actin gene, exon-1

carp β-actin promoter

Plasmid name: pFV4bCAT
Plasmid size: 6780 bp
Constructed by: Hackett lab
Construction date: 1992
Comments/References: CAT gene is cloned into BamHI site of the polycloning region.



Plasmid name: pFV5CAT
Plasmid size: 6780 bp
Constructed by: Hackett lab
Construction date: 1991
Comments/References: Not published yet. The same expression as in pFV2CAT.



CSGH polyA signal

CAT gene

carp β-actin gene, intron-1

carp β-actin gene, exon-1

carp β-actin promoter

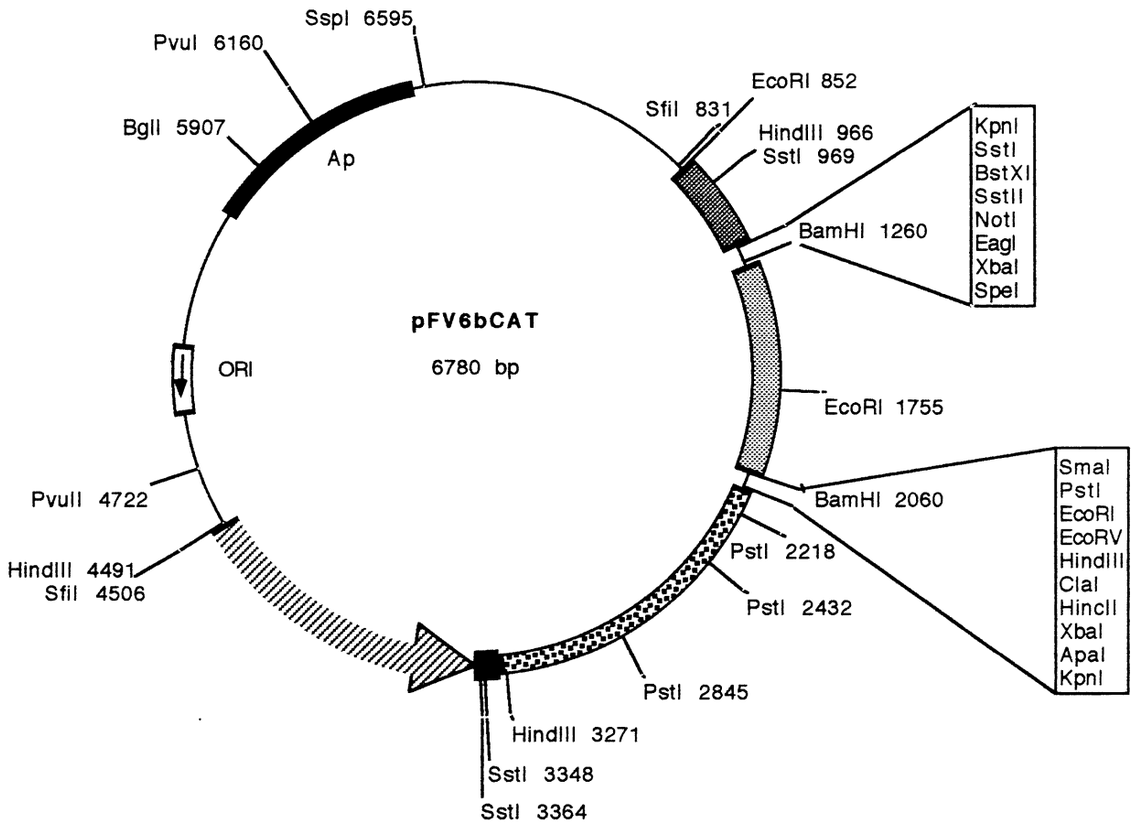
Plasmid name: pFV6aCAT

Plasmid size: 6780 bp

Constructed by: Hackett lab

Construction date: 1992

Comments/References: CAT gene is cloned into BamHI site of the polycloning region.



CSGH polyA signal

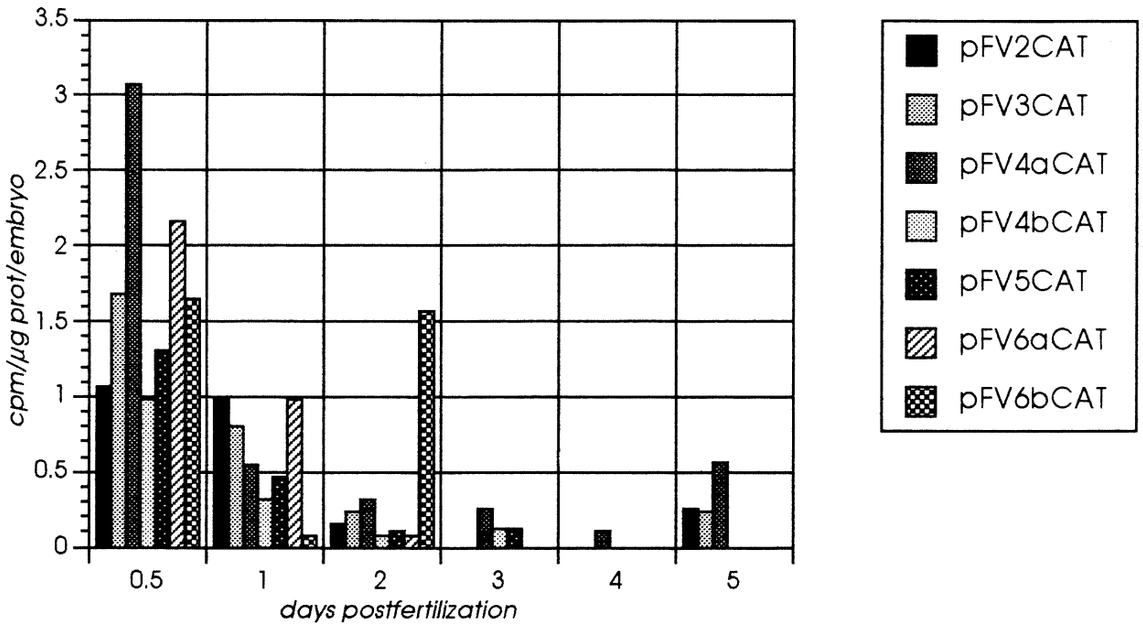
CAT gene

carp β-actin gene, intron-1

carp β-actin gene, exon-1

carp β-actin promoter

Plasmid name: pFV6bCAT
Plasmid size: 6780 bp
Constructed by: Hackett lab
Construction date: 1992
Comments/References: CAT gene is cloned into BamHI site of the polycloning region.


3.5
3
2.5
2
1.5
1
0.5
0
cpm/µg prot/embryo
0.5
1
2
3
4
5
days postfertilization
pFV2CAT
pFV3CAT
pFV4aCAT
pFV4bCAT
pFV5CAT
pFV6aCAT
pFV6bCAT

Gene, 108 (1991) 211–217

GENE 06133

Importance of the *CArG* box in regulation of β-actin-encoding genes

(Recombinant DNA; promoter; fish; carp; transcriptional control of expression)

Zhanjiang Liu[a], Boaz Moav[a,*], Anthony J. Faras[b,c], Kevin S. Guise[c,d†], Anne R. Kapuscinski[c] and Perry Hackett[a,c]

[a] Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108 (U.S.A.); [b] Department of Microbiology and [c] Institute of Human Genetics, University of Minnesota, Minneapolis, MN 55455 (U.S.A.), Tel. (612)624-9180; [d] Department of Animal Science, University of Minnesota, St. Paul, MN 55108 (U.S.A.), Tel. (612)624-4277; [e] Department of Fisheries and Wildlife, University of Minnesota, St. Paul, MN 55108 (U.S.A.), Tel. (612)624-3019

Received by J.L. Slightom: 6 December 1990
Revised/Accepted: 26 July/10 August 1991
Received at publishers: 3 September 1991

SUMMARY

The β-actin-encoding gene (*Act*) in carp is regulated by several *cis*-acting regulatory elements including the evolutionarily conserved $CC(A/T)_6GG$ (*CArG* box or serum-response element) sequences positioned in the promoter region between the CAAT and TATA boxes and in the first intron. To address the roles of the two *CArG* boxes on gene expression, we replaced them with linker sequences. The *CArG* box in the proximal promoter was not required for promoter activity in tissue-cultured cells, but was required in conjunction with a second *CArG* box in the first intron to give full expression in transgenic embryos. Likewise, the geometry of *cis*-acting transcriptional elements in the proximal promoter was more important for expression of transgenic constructs in developing embryos than in tissue-cultured fibroblasts. Mobility-shift and exonuclease mapping experiments indicated that the same or similar protein factors bind around the two *CArG* boxes, suggesting that interactions between the promoter and the first intron are involved in *Act* regulation.

INTRODUCTION

Genes are transcriptionally regulated by specific binding of *trans*-acting protein factors to *cis*-acting DNA regulatory sequences. Such interactions determine the tissue-specific, developmental stage-specific, and appropriate levels of expression. Thus, reporter genes linked to regulatory sequences of certain genes are generally expressed only in the cell lines or developmental stages in which the endogenous gene itself is transcribed (Atchison et al., 1989; Banerji et al., 1983; De Simone et al., 1987; Godbout et al., 1986; Okazaki et al., 1985; Wabl and Burrows, 1984; Zaller and Eckhardt, 1985) with some exceptions (Jantzen et al., 1987; Morgan et al., 1988). The *Act* genes are regulated by a number of protein factors which bind to *cis*-acting nt sequences in and around the transcriptional unit. The proximal promoter for the human *Act* gene contains a CAAT box, a TATA box, and a *CArG* motif in between the CAAT and TATA boxes. Two more *CArG* boxes have been found, one at 1400 bp upstream from the *tsp*, and the other in the first intron (Kawamoto et al., 1988). These elements are important for regulation of expression of this gene (Ng et al., 1989). A CAAT-binding factor has been identified (Frederickson et al., 1989) and an enhancer-binding factor has been shown to specifically associate with the enhancer

Correspondence to: Dr. P.B. Hackett, Department of Genetics and Cell Biology, 1445 Gortner Ave, University of Minnesota, St. Paul, MN 55108-1095 (U.S.A.)
Tel. (612)624-6736; Fax (612)625-5754.
* Permanent address: Department of Zoology, Tel Aviv University, Tel Aviv (Israel) Tel. (972)3-15459817.
† Deceased on 13 February 1991.

Abbreviations: aa, amino acid(s); *Act*, β-actin-encoding gene; bp, base pair(s); *CArG* box, $CC(A/T)_6GG$ sequence; *cat*, gene encoding Cm acetyltransferase; Cm, chloramphenicol; EPC, carp epithelial cells; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; *tsp*, transcription start point(s); u, unit(s); wt, wild type.

sequence in the first intron (Kawamoto et al., 1988). Additionally, the *CArG* boxes have been shown to bind serum-response factor (Frederickson et al., 1989) and to be inducible by serum and growth factors (Ng et al., 1989; Orita et al., 1989).

The human and carp *Act* genes are similar in organization and have similar regulatory sequences (Liu et al., 1989; 1990a, b). With carp *Act*, we have previously shown that (*i*) the proximal promoter is active by itself; (*ii*) the first intron contains positive regulatory sequences including the *CArG* motif that enhanced expression in an orientation-, position- and copy number-dependent manner, (*iii*) the first intron has a negative regulatory element in its 5′ region; (*iv*) the *CArG* and TATA boxes in the proximal promoter are required for promoter activity. Although possible interactions between *CArG* motifs in the proximal promoter and first intron have been suggested (Liu et al., 1990b), confirmation experiments have not been performed. Here we report that the proximal *CArG* motif is not required in tissue-cultured cells, but is required for expression in developing animals and that interactions between the promoter and intron *CArG* motifs appear to be important for regulation.

RESULTS AND DISCUSSION

(a) Function of the *CArG* motif within the proximal promoter

Our previous studies showed that deletion of the *CArG* motif together with the TATA box from the proximal promoter abolished promoter activity (Liu et al., 1990b). To determine whether the loss of promoter activity was due to the deletion of the *CArG* motif, the deletion of the TATA box, or both, we constructed a clone, pRC6dCArGCAT, containing all the proximal promoter elements except that the 29-bp sequence with the *CArG* box between the CAAT and TATA boxes replaced with a 29-bp linker sequence. An intermediate plasmid was generated in the process which had only a 25-bp linker sequence (Fig. 1). We included this construct in our experiments to gain insight into whether the spacing or geometry between the CAAT and TATA boxes is important. In prokaryotic promoters the geometry (facing) of *trans*-acting proteins bound to different sides of the DNA helix substantially affects transcriptional activity (Dunn et al., 1984; Irani et al., 1983; Lee and Schleif, 1989). The wt construct (pRC6CAT) was used as a control. Each construct was transfected into carp EPC cells and microinjected into fertilized zebrafish embryos, and transient expression of the *cat* marker gene was assayed. The results of the *cat* gene assays shown in Fig. 2 and summarized in Table I demonstrated that in cultured EPC cells, pRC6dCArGCAT gave as much *cat* gene activity as

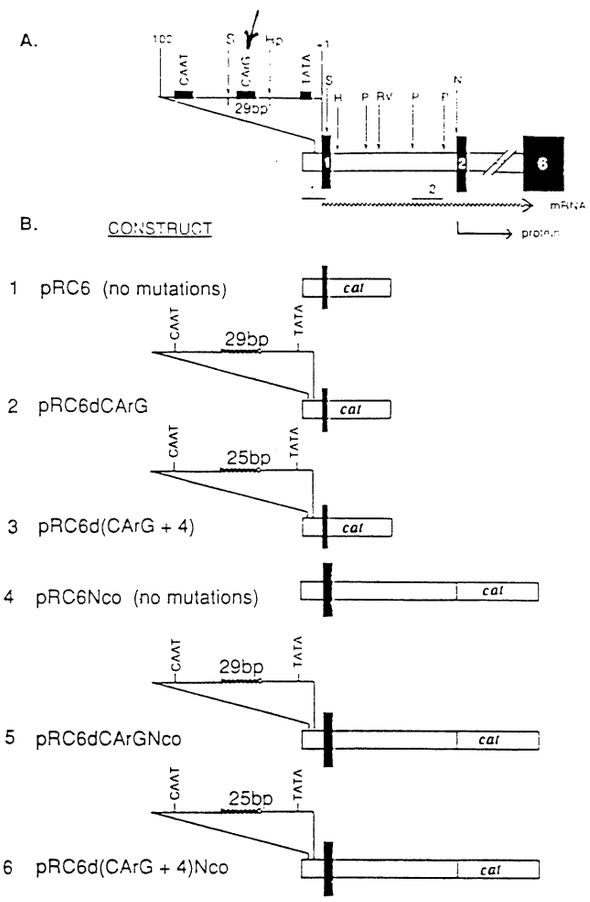


Fig. 1. Constructs used to determine *CArG* box function. (A) Organization of the carp β-actin gene with portion of exons 3–5 deleted. Blackened boxes, exons; unfilled rectangles, 5′-flanking sequence of 204 bp containing the proximal promoter elements (Liu et al., 1990b) and introns. Sa, *Sal*I; P, *Pst*I; S, *Sst*I; Hp, *Hpa*II; H, *Hin*dIII; RV, *Eco*RV; N, *Nco*I; +1, *tsp*; −100, 100 bp upstream from the *tsp*. The region between bp +1 and −100 has been expanded. The CAAT, *CArG*, and TATA boxes are indicated. The second line shows probes 1 and 2 (short lines numbered 1 and 2) used for mobility-shift assays (Figs. 3 and 4A). (B) Wavy lines, linker replaced regions; the numbers indicate linker lengths. All constructs contained the *cat* reporter gene. To make the *CArG* deletion clones, the *Sst*I fragment of pRC6 (Liu et al., 1990b) was cloned into the *Sst*I site of pUC119 to make pRC6-5′. Clone 3 was constructed by cloning the *Msp*I/*Hin*dIII fragment (the *Msp*I site is located 8 bp downstream from the *CArG* motif) of pRC6 into the *Acc*I/*Hin*dIII sites of pRC6-5′; pRC6d(CArG + 4), is identical to pRC6 except that 29 bp containing the *CArG* motif between the CAAT and TATA boxes was replaced by a 25-bp polylinker sequence in pUC119 from *Sst*I–*Acc*I site. To obtain a plasmid with the same spacing between the CAAT and TATA boxes, clone 3 was digested with *Bam*HI in the replaced polylinker sequence, filled-in by PolIk (BRL) and recircularized, generating clone 2. Clones 4, 5, and 6 were constructed by inserting the corresponding proximal promoter into the *Sal*I site of pSalNcoCAT (Liu et al., 1990b).

the wt pRC6CAT, indicating that the TATA box is required for expression, but that the *CArG* box in the proximal promoter is not. In addition, a decrease in spacing between the

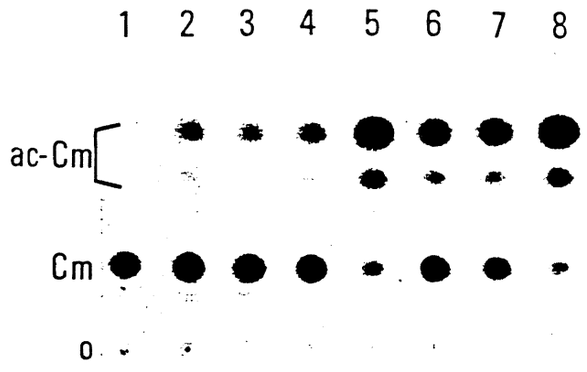


Fig. 2. Typical *cat* gene assays from EPC-transfected cells (Liu et al., 1990c). Lanes: **1**, promoterless control; **2**, clone 1 (Fig. 1B); **3**, clone 2; **4**, clone 3; **5**, clone 4; **6**, clone 5; **7**, clone 6; **8**, 0.07 u of pure *cat* gene enzyme (Sigma). Half the amount of cell extract for pRC6NcoCAT was used in lane **5**. **o**, origin; **Cm**, [^{14}C]-Cm; **ac-Cm**, acetylated forms of [^{14}C]Cm. Carp EPC were transfected by the Ca·phosphate precipitation method (Graham and Van der Eb, 1973) with modifications (Chu and Sharp, 1981). From 30–50% confluent plates of EPC cells were transfected with 24 μg DNA/100 mm dish. Sixty hours after transfection, cells were harvested for analysis of enzymatic activities (Liu et al., 1990b).

CAAT and TATA boxes by 4 bp (pRC6d[CArG + 4] CAT) which alters the rotational geometry by about 140° did not appreciably affect the expression, thereby suggesting that the spacing and facing between the two boxes are not critical for expression in tissue-cultured fish cells.

TABLE I

Expression of *cat* gene constructs[a]

Constructs	*cat* activity	
	Tissue culture	Fish
pCAT	0.1	0.4
pRC6CAT	2	22
pRC6dCArGCAT	2	12
pRC6d[CArG + 4]CAT	3	2
pRC6NcoCAT	100	100
pRC6dCArGNcoCAT	10	27
pRC6d[CArG + 4]NcoCAT	16	2

[a] Expression of *cat* gene in EPC cells and in developing fish. For EPC analysis, four plates were used for each construct and enzyme activities were averaged; the variation between assays was less than 10%. The values were normalised to that of pRC6NcoCAT. For developing embryos, samples of 30 transgenic fish were taken for *cat* gene analysis each day after microinjection for seven days. The values for each construct were integrated over the seven-day period. The values in both columns were normalized to that of pRC6NcoCAT (100% = 0.02 u in tissue culture, 0.004 u in transgenic fish). Microinjection into the central position of the germinal disc of the fertilized eggs of zebrafish was carried out as described (Liu et al., 1990c).

To address the function of the evolutionarily conserved *CArG* motif in the developing animal, the DNA constructs were each microinjected into 500 fertilized zebrafish eggs. Samples of 30 eggs were randomly taken from each injected batch daily after injection for seven days (days two–eight postinjection). Assays of *cat* gene activity were performed to monitor *cat* gene expression and the total integrated expression was calculated (Table I). The wt proximal promoter (pRC6CAT) directed *cat* gene activity at only twice the rate of the *CArG*$^-$ clone (pRC6dCArGCAT), indicating that the *CArG* motif is required for full promoter activity. However, the spacing/facing between the CAAT and TATA boxes appeared to be important in the developing animal for expression. The expression of PRC6d[CArG + 4]CAT was 9% that of pRC6CAT, suggesting that alteration of the geometry between the CAAT and TATA boxes is essential for full transcriptional activity. In several S1-nuclease and RNase-protection assays for the different mRNAs from the transgene constructs we were unable to detect *cat* mRNAs, which must exist at levels less than 0.5% that of endogeneous *Act* mRNAs in zebrafish embryos (Z.L., unpublished observation). Accordingly, our conclusions are based on the assumption that transcriptional initiation is correct in fish embryos as it is in transfected fish cells (Liu et al., 1990b).

(b) Deletion of the *CArG* motif within the proximal promoter reduces enhancement by intron elements

Our previous results (Liu et al., 1990b) suggested interactions between the *CArG* sequences in the proximal promoter and the first intron might regulate *Act* gene expression. Thus, if interactions between the two *CArG* boxes are essential for function, deletion of the *CArG* box within the proximal promoter should result in loss of enhancement of expression by the intronic element. To test our hypothesis, we made the constructs numbered 4–6 (Fig. 1B) by linking intron-1 of the *Act* gene to constructs 1–3 (Fig. 1B) and either transfected them into carp EPC cells or microinjected them into fertilized zebrafish embryos. Expression of the *cat* gene was determined for each construct. In the cultured cells, although no effect was observed with deletion of the proximal *CArG* box for promoter activity (constructs 1 and 2 above), a tenfold difference in *cat* gene activity was observed between the wt promoter plus intron (pRC6NcoCAT) and the *CArG*$^-$ promoter plus intron (pRC6dCArGNcoCAT) (Fig. 2 and Table I). The 4-bp space alteration between the CAAT and TATA boxes did not further reduce the expression of pRC6dCArGNcoCAT (Fig. 2 and Table I). However, in the developing animal, deletion of the proximal *CArG* motif reduced activity fourfold and alteration of the spacing further lowered expression another tenfold. These differences in effects in tissue-cultured cells and the developing animal may be due

to differences in the presence, concentration and/or quality of the *trans*-acting protein factor(s). Similar results showing differences in relative expression in tissue-cultured cells and transgenic animals have been obtained with rat albumin (Heard et al., 1987; Herbomel et al., 1989; Tronche et al., 1989; Pinkert et al., 1987) and elastase I (Swift et al., 1989) genes.

(c) Specific interaction of cell nuclear factors with β-actin gene regulatory sequences

We used two probes for mobility shift assays (Fried and Crothers, 1981) to detect the protein factors which could be involved in the regulation of β-actin gene expression. Probe 1, the proximal promoter bp −204 to bp +68, contained the CAAT, *CArG*, and TATA boxes that comprised the regulatory elements of the pRC6 clone (Fig. 1A). In the experiment shown in Fig. 3, nuclear extracts were prepared from the EPC cells and mobility-shift binding reactions were performed. In absence of the nuclear extract, the 3′-labelled fragment migrated as a discrete band (lane 1). When the crude nuclear extract was added to the binding reaction, a single, major, shifted band was prominent (lane 2). The specificity of the binding was confirmed by competition tests. When either a 25-fold or 50-fold molar excess of unlabelled probe fragment was included, the intensity of the shifted band decreased (lanes 3 and 4). To resolve further which sequences in the 272-bp probe the protein factor bound, we used 25-fold and 50-fold molar excesses of the unlabelled intron fragment containing the *CArG* motif as a competitor. As shown in Fig. 3 (lanes 5 and 6), the intronic fragment competed as well as the proximal promoter fragment. This suggested that the protein factor(s) was binding to the *CArG* motif. This conclusion was supported by the inability of the promoter fragment lacking the *CArG* box to compete with the protein

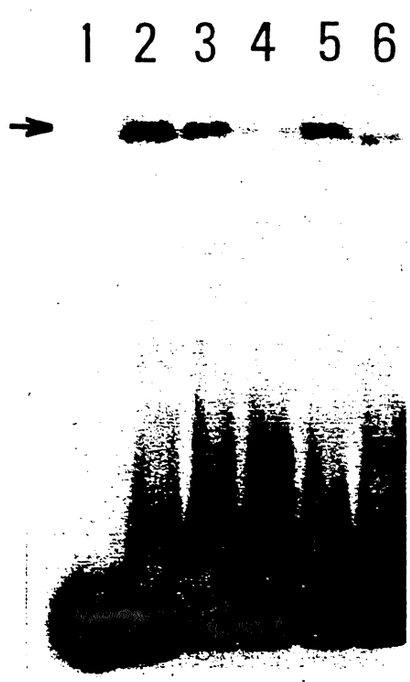


Fig. 3. Sequence-specific binding of protein factors to the promoter region as revealed by a mobility shift assay. Plasmid pRC6 fragment (probe 1, Fig. 1A) was end-labelled. Nuclear extract was derived from EPC cells. Plasmid pUC119 digested with *Msp*I and poly(dI/dC) were used as a nonspecific competitor. Lanes: **1**, no extract; **2–6**, assays contain 10 μg (Bradford, 1976) of nuclear extract; **2**, no specific competitor; **3** and **4**, with 25-fold and 50-fold molar excess of unlabelled pRC6 fragment as competitor, respectively; **5** and **6**, with 25-fold and 50-fold molar excess of unlabelled probe 2 fragment (Fig. 1A). Carp EPC cell nuclear extracts were prepared according to Dignam et al. (1983) with 2 μg/ml of the protease inhibitor leupeptin and aprotinin. The 20 μl binding reactions (Gustafson et al., 1988) contained 2 μg pUC119 and 2 μg poly(dI/dC) as non-specific competitors, 5–10 μg of crude nuclear extracts (Bradford, 1976), 0.1–1.0 ng of 3′-end labelled probe, and appropriate specific competitors. After incubation at room temperature for 10 min the reaction mixtures were loaded onto 6% (29:1) polyacrylamide-bisacrylamide gels.

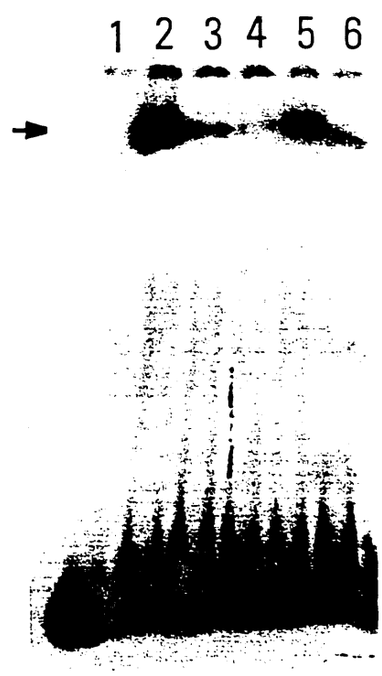


Fig. 4. Mobility shift assay with the fragment of the first intron containing the positive regulatory element (probe 2, Fig. 1A). Conventions are the same as in Fig. 3. Lanes: **1**, no extract; **2–6**, with 10 μg of the nuclear extract each. Lanes: **2**, no specific competitor; **3** and **4**, with 25-fold and 50-fold molar excess of the unlabelled intron fragment (probe 2) as competitor; **5** and **6**, with 25-fold and 50-fold molar excess of the unlabelled probe 1 fragment as competitor.

factor binding (data not shown). The competition tests indicated that the protein factors bound at the *CArG* boxes in the proximal promoter and in the intron are probably similar factors. The results left open the question whether the same or similar proteins bind to the two *CArG* sequences in a fashion analogous to the CAAT-binding protein gene family (Raymondjean et al., 1988).

The second probe we used for identification of *trans*-acting regulators was the intronic fragment used above as competitor (Fig. 1A). As with probe 1, the addition of nuclear extract to the binding reaction gave a single shifted band (Fig. 4, lane 2) whose specificity was confirmed by competition with 25-fold and 50-fold molar excesses of unlabelled probe 2 fragment. Probe 1 fragment was able to compete, but not as well, on a molar basis, as the intronic fragment.

We only detected a single shifted band with the proximal promoter probe although at least two more known regulatory motifs are located in this fragment. To confirm the protein factors bind at the *CArG* box in the proximal promoter as shown with competition test, we performed exonuclease mapping. The probe was 3′-labelled at one end downstream from the gene. After the binding reaction was completed, phage λ exonuclease was added which digests from the 5′ ends of linear DNA. If a protein binds to a certain region, the exonuclease should stop at the site where the protein factor binds. As shown in Fig. 5, a fragment of 151 bp was protected. The 5′ end of the protected fragment matched precisely the 5′ end of the *CArG* box in the proximal promoter, demonstrating that the protein factor we detected with probe 1 bound at the *CArG* box. Additional DNaseI protection experiments were conducted which showed that the *CArG* region was protected by extracts in the nuclear extracts described above (Z.L., unpublished observation).

Fig. 5. Exonuclease mapping. S, sequencing ladder of pUC119 polylinker sequence with G, A, T. Lanes: **1**, probe 1 (upper arrow) as shown in Fig. 1A; **2**, probe 1 with phage λ exonuclease treatment; **3**, probe 1 with 10 μg of the nuclear extract and treated with phage λ exonuclease; lower arrow, 151-bp protected fragment. The binding reaction was performed as described in Fig. 3 after which 2 μl of 10 × buffer (670 mM glycine-KOH pH 9.4/25 mM $MgCl_2$) and 5 u of phage λ exonuclease (BRL) were added. The reaction was incubated at 37°C for 10 min and stopped by phenol extraction. The extracted DNA was analyzed by electrophoresis on a sequencing gel.

(d) Conclusions

Consistent with the synergistic function of the proximal promoter and the intronic elements, mobility-shift competition tests (Figs. 3 and 4) indicated that the same class of *trans*-acting protein factor(s) bind both regions with *CArG* boxes which favours a DNA looping mechanism (Ptashne, 1988). The interactions between the *CArG* boxes may be accomplished by the same factor. When the *CArG* box in the proximal promoter was deleted, the interaction cannot occur and transcriptional activity was low. When the intronic element containing the *CArG* box was inverted expression was low (Liu et al., 1990b). The data suggest that either promoter and intronic interactions may still occur at the cost of disrupting the normal spacial geometry of the transcriptional complex (Bornstein et al., 1988) or that the interaction might not occur due to an altered stereostructure (see Takahashi et al., 1986). Similarly, interactions between two *CArG* boxes in the proximal regulatory region of the human cardiac α-acting-encoding gene (Miwa and Kedes, 1987), and between elements in the promoter and enhancer of the prolactin-encoding gene (Crenshaw et al., 1989) and the *rpL32* gene (Atchison et al., 1989;

Chung and Perry, 1989), have been suggested to account for the synergistic functions of similar elements.

ACKNOWLEDGEMENTS

We thank Mark Dalton for preparing the figures and Aris Moustakas for discussions. This work was sponsored by grants from the NIH and the Minnesota Sea Grant College Program (paper JR259) supported by the NOAA Office of Sea Grant, Department of Commerce, under Grant No. NOAA-86AA-D-SG112-project R/A-6. Z.L. was supported by a stipend from Minnesota Sea Grant.

REFERENCES

Atchison, M.L., Meyuhas, O. and Perry, R.P.: Localization of transcriptional regulatory elements and nuclear factor binding sites in mouse ribosomal protein gene rpL32. Mol. Cell. Biol. 9 (1989) 2067–2074.

Banerji, J., Olson, L. and Schaffner, W.: A lymphocyte-specific cellular enhancer is located downstream of joining region in immunoglobulin heavy chain genes. Cell 33 (1983) 729–740.

Bornstein, P., McKay, J., Liska, D., Apone, S. and Devarayalu, S.: Interactions between the promoter and first intron are involved in transcriptional control of alpha-1(I) collagen gene expression. Mol. Cell. Biol. 8 (1988) 4851–4857.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72 (1976) 248–254.

Chu, G. and Sharp, P.A.: SV40 DNA transfection of cells in suspension: analysis of the efficiency of transcription and translation of T-antigen. Gene 13 (1981) 197–202.

Chung, S. and Perry, R.P.: Importance of introns for expression of mouse ribosomal protein gene rpL32. Mol. Cell. Biol. 9 (1989) 2075–2082.

Crenshaw III, E.B., Kalla, K., Simmons, D.M., Swanson, L.W. and Rosenfeld, G.: Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. Genes Develop. 3 (1989) 959–972.

De Simone, V., Giliberto, G., Hardon, E., Paonessa, G., Palla, F., Lundberg, L. and Cortese, R.: *Cis* and *trans*-acting elements responsible for cell-specific expression of the human alpha-1-antitrypsin gene. EMBO J. 6 (1987) 2759–2766.

Dignam, J.D., Lebowitz, R.M. and Roeder, R.G.: Accurate transcription initiated by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11 (1983) 1475–1489.

Dunn, T.M., Hahn, S., Ogden, S. and Schlief, R.F.: An operator at −280 base pairs that is required for repression of *araBAD* operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc. Natl. Acad. Sci. USA 81 (1984) 5017–5020.

Frederickson, R.M., Micheau, M.R., Iwamoto, A. and Miyamoto, N.G.: 5′ flanking and first intron sequences of the human β-actin gene required for efficient promoter activity. Nucleic Acids Res. 17 (1989) 253–270.

Fried, M. and Crothers, D.M.: Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. 9 (1981) 6505–6525.

Godbout, R., Ingram, R. and Tilghman, S.M.: Multiple regulatory elements in the intergenic region between the α-fetoprotein and albumin genes. Mol. Cell. Biol 6 (1986) 477–487.

Graham, F.L. and Van der Eb, A.J.: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52 (1973) 456–467.

Gustafson, T.A., Miwa, T., Boxer, L.M. and Kedes, L.: Interaction of nuclear proteins with muscle-specific regulatory sequences of the human cardiac α-actin promoter. Mol. Cell. Biol. 8 (1988) 4110–4119.

Heard, J.M., Herbomel, P., Ott, M.O., Mottura, A., Weiss, M. and Yaniv, M.: Determinants of the rat albumin promoter tissue specificity analyzed by an improved transient expression system. Mol. Cell. Biol. 7 (1987) 2425–2434.

Herbomel, P., Rollier, A., Tronche, F., Ott, M.-O., Yaniv, M. and Weiss, M.C.: The rat albumin promoter is composed of six distinct positive elements within 130 nucleotides. Mol. Cell. Biol. 9 (1989) 4750–4758.

Irani, M.H., Oroszand, L. and Adhya, S.: A control element within a structural gene: the *gal* operon of *Escherichia coli*. Cell. 32 (1983) 783–788.

Jantzen, H.M., Strahle, U., Gloss, B., Stewart, F., Schmidt, W., Bohart, M., Miksicek, R. and Schutz, G.: Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell. 49 (1987) 29–38.

Kawamoto, T., Makino, K., Niwa, H., Sugiyama, H., Kimura, S., Amemura, M., Nakata, A. and Kakunaga, T.: Identification of the human β-actin enhancer and its binding factor. Mol. Cell. Biol. 8 (1988) 267–272.

Lee, D.-H. and Schleif, R.F.: In vivo DNA loops in *araBAD*: size limits and helical repeat. Proc. Natl. Acad. Sci. USA 86 (1989) 476–480.

Liu, Z., Zhu, Z., Roberg, K., Faras, A., Guise, K., Kapuscinski, A. and Hackett, P.: The β-actin gene of carp (*Ctenopharyngodon idella*). Nucleic Acids Res. 17 (1989) 5850.

Liu, Z., Zhu, Z., Roberg, K., Faras, A., Guise, K., Kapuscinski, A. and Hackett, P.B.: Isolation and characterization of the β-actin gene of carp (*Cyprinus carpio*). DNA Sequence-J. DNA Seq. Map. 1 (1990a) 125–136.

Liu, Z., Moav, B., Faras, A., Guise, K., Kapuscinski, A. and Hackett, P.B.: Functional analysis of the control elements that affect expression of the β-actin gene of carp. Mol. Cell. Biol. 10 (1990b) 3432–3440.

Liu, Z., Moav, B., Faras, A., Guise, K., Kapuscinski, A. and Hackett, P.B.: Development of vectors for transgene expression in fish. Bio/Technology 8 (1990c) 1268–1272.

Miwa, T. and Kedes, L.: Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. 7 (1987) 2803–2813.

Morgan, J.G., Courtois, G., Fourel, G., Chodosh, L.A., Campbell, L., Evans, E. and Crabtree, G.R.: SP1, a CCAAT-binding factor, and the adenovirus major late promoter transcription factor interact with functional regions of the γ-fibrinogen promoter. Mol. Cell. Biol. 8 (1988) 2628–2637.

Ng, S.-Y., Gunning, P., Lui, S.-H., Leavitt, J. and Kedes, L.: Regulation of the human β-actin promoter by upstream and intron domains. Nucleic Acids Res. 17 (1989) 601–615.

Okazaki, K., Yasuda, K., Kondoh, H. and Okada, T.S.: DNA sequences responsible for tissue-specific expression of a chicken alpha-crystallin gene in mouse lens cells. EMBO J. 4 (1985) 2589–2595.

Orita, S., Makino, K., Kawamoto, T., Niwa, H., Sugiyama, H. and Kakunaga, T.: Identification of a site that mediates transcriptional response of the human β-actin gene to serum factors. Gene 75 (1989) 13–19.

Pinkert, C., Ornitz, D., Brinster, R. and Palmiter, R.: Albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Develop. 1 (1987) 268–277.

Ptashne, M.: How eukaryotic transcriptional activators work. Nature 335 (1988) 683–689.

Raymondjean, M., Ceregnini, S. and Yaniv, M.: Several CCAAT binding proteins coexist in eukaryotic cells. Proc. Natl. Acad. Sci. USA 85 (1988) 757–761.

Swift, G.H., Kruse, F., MacDonald, R.J. and Hammer, R.E.: Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes Develop. 3 (1989) 687–696.

Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M. and Chambon, P.: Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature 319 (1986) 121–126.

Tronche, F., Rollier, A., Bach, I., Weiss, M.C. and Yaniv, M.: The rat albumin promoter: cooperation with upstream elements is required when binding of APF/HNF1 to the proximal element is partially impaired by mutation or bacterial methylation. Mol. Cell. Biol. 9 (1989) 4759–4766.

Wabl, M. and Burrows, P.: Expression of immunoglobulin heavy chain at high level in the absence of a proposed immunoglobulin enhancer element in *cis*. Proc. Natl. Acad. Sci. USA 81 (1984) 2452–2455.

Zaller, D. and Eckhardt, L.: Deletion of a B-cell-specific enhancer affects transfected, but not endogenous, immunoglobulin heavy-chain gene expression. Proc. Natl. Acad. Sci. USA 82 (1985) 5088–5092.

Aquaculture, 103 (1992) 253–273
Elsevier Science Publishers B.V., Amsterdam

Molecular analysis and growth evaluation of northern pike *(Esox lucius)* microinjected with growth hormone genes

Mark L. Gross[a,*], John F. Schneider[c,d,*], Neomi Moav[b,i], Boaz Moav[e,f], Carmen Alvarez[b,g], Steve H. Myster[d], Zhanjiang Liu[e], Eric M. Hallerman[h], Perry B. Hackett[d,e], Kevin S. Guise[b,d], Anthony J. Faras[c,d] and Anne R. Kapuscinski[a]

[a]*Fisheries and Wildlife,* [b]*Departments of Animal Science,* [c]*Microbiology,* [d]*The Institute of Human Genetics, and* [e]*Genetics and Cell Biology, University of Minnesota, St. Paul, MN, USA*
[f]*Department of Zoology, Tel-Aviv University, Ramat-Aviv, Israel*
[g]*Departamento de Biologia Celular y Genetica, Universidad de Malagas, Campus Universitario de Teatinos, Malaga, Spain*
[h]*Department of Fisheries and Wildlife Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*
[i]*Biotechnology General Kiryat Weizmann, Rehovot, Israel*

(Accepted 11 September 1991)

ABSTRACT

Gross, M.L., Schneider, J.F., Moav, N., Moav, B., Alvarez, C., Myster, S.H., Liu, Z., Hallerman, E.M., Hackett, P.B., Guise, K.S., Faras, A.J. and Kapuscinski, A.R., 1992. Molecular analysis and growth evaluation of northern pike *(Esox lucius)* microinjected with growth hormone genes. *Aquaculture*, 103: 253–273.

Bovine (bGH) or chinook salmon *(Oncorhynchus tshawytscha)* growth hormone (csGH) cDNA genes were transferred by microinjection into newly fertilized northern pike *(Esox lucius)* eggs. Non-lethal screening of fin tissue showed genomic integration of transgenes in 88 of 1398 putative transgenic fish. Expression of bGH transgenes under transcriptional control of the Rous sarcoma virus long terminal repeat was detected in 36 of 1218 putative transgenic fish examined by radioimmunoassay of blood serum. Bovine growth hormone was also detected in mesodermal tissue of fins from microinjected fish using thin slice immunohistochemistry. Southern hybridizations of six tissues from a sample of 40 microinjected individuals revealed a high degree of mosaicism, with 30% of the fish containing detectable transgenic DNA in one or more tissues and only 41% of these containing detectable transgenes in fins.

Growth of microinjected fish was quantitatively evaluated in three experiments. Average weight of microinjected fish was greater than that of controls of the same sex in four out of six groups. Significant growth enhancement ($P<0.05$) was detected only for microinjected males in one experiment. Comparisons among molecular assays and individual fish growth in the founder generation indicated that the high degree of mosaicism prevented non-lethal indentification of all transgenic individuals and influences detection of growth enhancement.

Correspondence to: Dr. A.R. Kapuscinski, Fisheries and Wildlife, University of Minnesota, St. Paul, MN 55108, USA.
*Authors contributed equally; order was determined by a coin toss.

INTRODUCTION

The creation of growth-enhanced mice using gene transfer (Palmiter et al., 1982) has sparked interest in applying these techniques to improve traits in fish. While the classical method of improving traits is to develop a genetic selection program, the application of gene transfer has the potential to further enhance traits such as growth, disease resistance, and feed efficiency. Gene transfer experiments in fish have concentrated on growth enhancement using growth hormone genes (Guise et al., 1990; Kapuscinski and Hallerman, 1990). In some mammals containing growth hormone transgenes, increased growth hormone levels have caused deleterious perturbations in their endocrine balance resulting in serious side effects. For example, uncontrolled expression of growth hormone in pigs produced developmental problems that ranged from diabetes and infertility to lethality (Pursel et al., 1989). Fish, however, may have the greatest promise for the action of transferred growth hormone genes since their rate of growth has been shown to be highly responsive to injections of crude, purified, or recombinant-derived growth hormone protein (Adelman, 1977; Agellon and Chen, 1986; Weatherly and Gill, 1987a,b).

Landings from the world's fisheries are rapidly approaching the predicted maximum sustainable yield for natural waters. This trend has contributed to the increasing importance of aquaculture production (Stickney, 1990). Because many aquaculture operations utilize wild or recently domesticated stocks, genetic improvements could potentially be financially beneficial. Several research groups have successfully introduced growth hormone genes into fish (Zhu et al., 1985; Chourrout et al., 1986; Dunham et al., 1987; Rokkones et al., 1989) with a few noting increased growth in the treated group (Zhu et al., 1986; Zhang et al., 1990). The latter reports, however, presented limited information about rearing conditions and statistical analyses, making it difficult to quantitatively evaluate conclusions about the observed growth enhancement. In conjunction with molecular analyses, we therefore initiated growth studies under carefully controlled rearing conditions to quantitatively evaluate the performance of transgenic fish containing extra growth hormone genes.

MATERIALS AND METHODS

DNA constructs

A fish expression vector (FV), FV-2/csGH, was constructed by inserting the fragment containing chinook salmon *(Oncorhynchus tshawytscha)* growth hormone cDNA (Hew et al., 1989) into the *Eco*RI site of p*HIN/Nco* (Liu et al., 1990a). Thus, FV-2/csGH contains the proximal promoter and enhancer regulatory elements of the common carp *(Cyprinus carpio)* β-actin gene. To

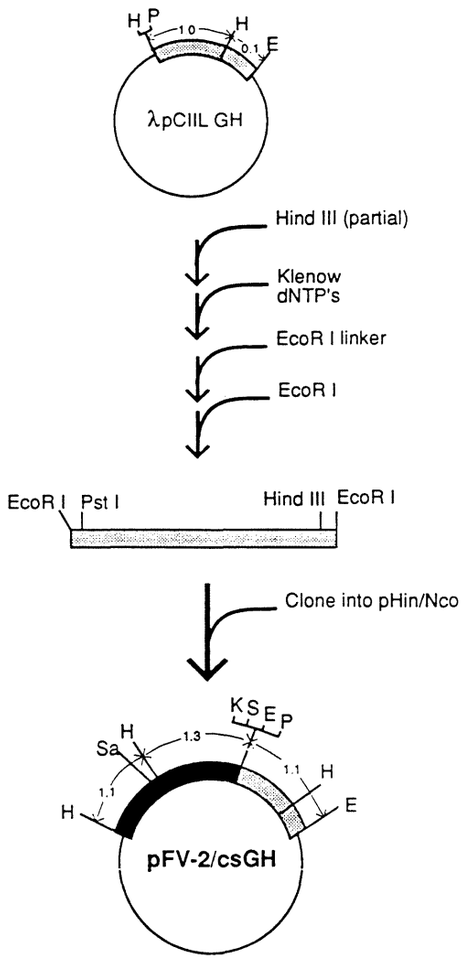


Figure Legend:

E, EcoR I; H, Hind III; K, Kpn I; P, Pst I; S, Sst I; Sa, Sal I

carp ß- actin sequence chinook salmon GH cDNA sequence

vector sequences

Fig. 1. Construction of FV-2/csGH. Restriction sites are as indicated.

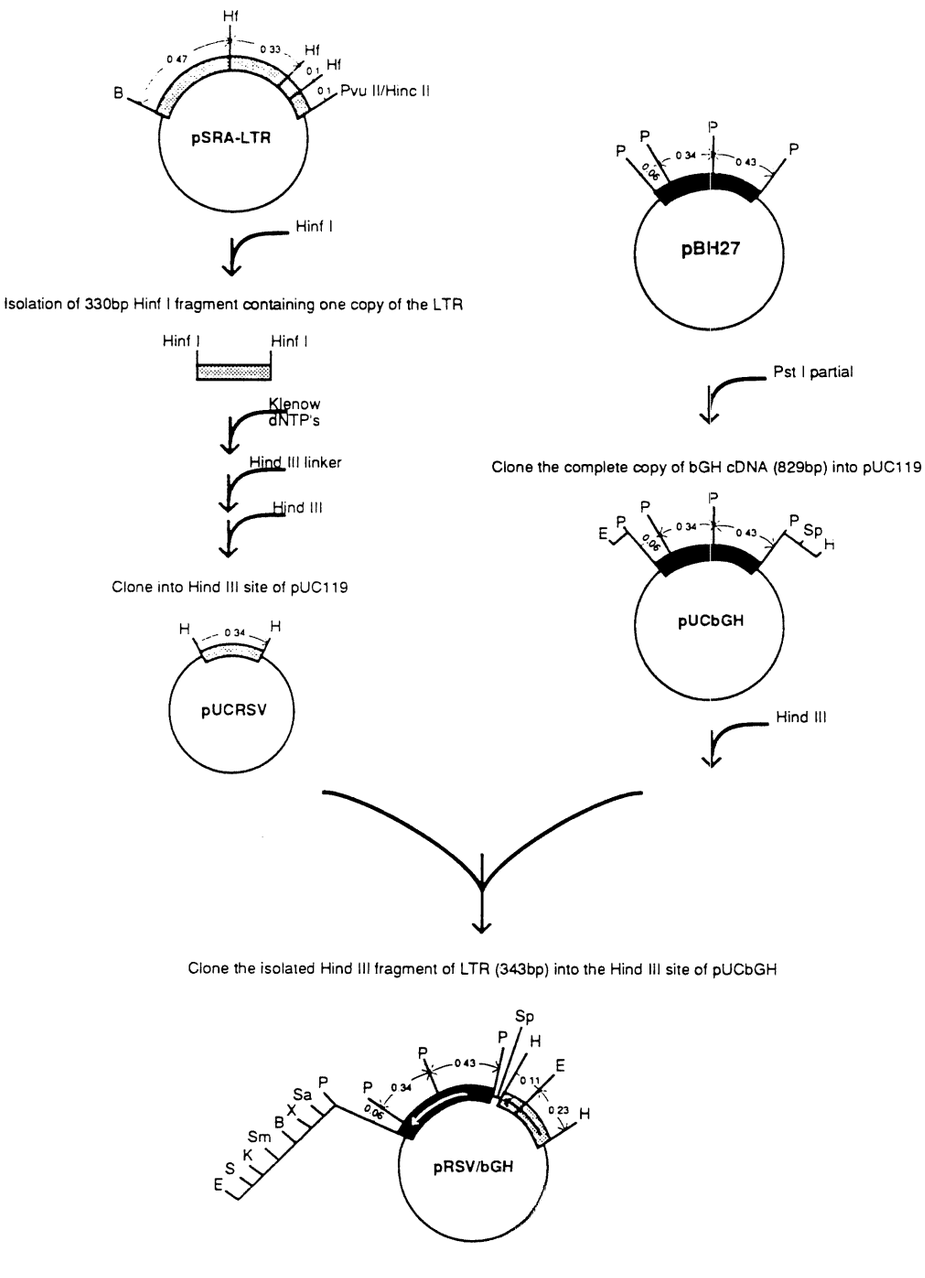


Fig. 2. Construction of pRSV/bGH. Restriction sites are as indicated.

insert the cDNA fragment into the *Eco*RI site of p*Hin/Nco,* the clone that harbors the cDNA for csGH was partially digested with *Hin*dIII, filled-in with Klenow fragment DNA polymerase I, ligated to *Eco*RI linkers and digested with *Eco*RI endonuclease (Fig. 1).

To construct pRSV/bGH, the 0.83 Kb cDNA of bovine growth hormone was cloned into the *Pst*I site of pUC119 by partial digestion of pBH27 (provided by Hank George, Molecular Genetics Inc., Edina, MN) with *Pst*I to produce pUCbGH. The Rous Sarcoma virus (RSV) promoter contained within a 0.33 Kb *Hin*fI fragment (Yoon et al., 1990) was blunted with the Klenow fragment of polymerase I. *Hin*dIII linkers were added and this fragment was cloned into the *Hin*dIII site of pUCbGH to yield pRSV/bGH (Fig. 2).

Gamete collection, fertilization, and microinjection

Northern pike *(Esox lucius)* gametes were collected from wild individuals and transported at 4°C to the lab. Eggs from 2–3 females were mixed and fertilized with sperm from 2–3 males. A portion of the fertilized eggs was randomly allocated for controls. Twenty minutes after fertilization, a smoothly tapered borosilicate needle with an inner tip diameter of approximately 2 μm (Yoon et al., 1990) was used to microinject approximately 50 picoliters of linearized DNA solution into the germinal disc directly through the chorion and vitelline membranes. The plasmid pRSV/bGH was linearized with the restriction endonuclease *Kpn*I, extracted with phenol/chloroform, precipitated in ethanol, and re-dissolved in 88 m*M* NaCl, 10 m*M* Tris·HCl, pH 7.6 to a final concentration of 25 ng/μl. The plasmid FV-2/csGH was microinjected in the superhelical form at the same final concentration. Microinjection proceeded under a stereo microscope with the borosilicate needle attached to a Brinkman MM33 micromanipulator and the amount of plasmid solution injected was controlled by a Medical Systems Corporation pico-injector (Model PLI-100).

DNA analysis

The physical state of the transgene DNA was characterized by Southern blot hybridization analysis, as described by Hallerman et al. (1990). DNA was isolated from individual 6-day-old embryos or juvenile fish fin tissues by sodium dodecyl sulfate lysis (SDS) and proteinase K digestion. DNA (7 μg) was digested with excess *Bam*HI restriction endonuclease, electrophoresed through a 0.8% agarose gel, transferred onto nylon membranes by Southern blotting and then probed for the presence of transgenic DNA. The entire plasmid, either pRSV/bGH or FV-2/csGH, was labeled with ^{32}P by the random priming method.

For two-dimensional gel electrophoresis, uncut and cellular DNA was electrophoresed first in 0.4% agarose and then at right angles in 1.2% agarose,

transferred to nylon membranes, and hybridized to labeled pRSV/bGH DNA (Manias et al., 1989). This method separates open circular and superhelical extra-chromosomal transgene DNA, both unit length and multimers, from linear cellular and integrated transgene DNA (Manias et al., 1989).

Growth hormone radioimmunoassay

Blood serum samples collected from dorsal aortae of 2-month-old fish were analyzed for the presence of bGH protein by radioimmunoassay. Because the pRSV/bGH construct included the signal peptide sequence, detection of bGH protein was expected. Radioiodinated bGH was prepared by adding four iodo-Beads (Pierce Chemical Co., Rockford, IL) to a vial containing 1.5 mCi ^{125}I and 0.2 ml buffer (0.25 *M* Na_3PO_4 pH 7.5). After 5 min, 15 μg bovine growth hormone (USDA) solubilized in 0.01 *M* $NaHCO_3$ diluted with 20 μl of buffer was added and incubated for 15 min. The mixture was purified by column chromatography using Bio-gel P-30 (Bio-Rad) equilibrated and eluted with 0.1% bovine serum albumin (BSA)–barbitol buffer pH (Sigma) as three peaks. The middle peak was diluted in 1% BSA–0.01 *M* phosphate-buffered saline (PBS) pH 7.5. Anti-serum to ovine GH (NIAMDD-anti-oGH) was diluted 1:5000 in 1% normal rabbit serum–0.05 *M* EDTA-PBS pH 7.0, and 0.1 ml dispensed into assay tubes containing 0.3 ml 1% egg albumin–PBS pH 7.0 and 0.3 ml test plasma. After 24 h, 0.1 ml tracer (20 000 cpm) was added, followed 24 h later by the addition of 0.1 ml precipitating antiserum (sheep anti-rabbit gamma globulin) and incubation for 48 h. Samples were centrifuged at 15 000$\times g$ for 20 min and counted in a Beckman gamma counter. Assay sensitivity was approximately 1 ng/tube, equivalent to 1.1 ng/ml (Wheaton et al., 1986).

Immunohistochemical detection of tissues expressing bGH

Fin tissue collected from individual fish in the microinjected groups was fixed, sectioned, and mounted on slides using standard clinical techniques. Slides were deparafinized by a 30 min room temperature dip in xylol, followed by two 5 min dips in 100%, 90%, and 70% ethanol. Slides were washed three times in PBS pH 7.6 for 10 min, dipped in a 0.3% H_2O_2 in PBS for 30 min, and rewashed three times in PBS for 5 min. Blocking was by incubation in a 1$\times$dilution of normal sheep serum for 30 min at 37°C, followed by a gentle wiping. Slides were incubated in polyclonal rabbit anti-bGH diluted 1:100 in normal sheep serum overnight at 4°C, washed three times in PBS for 10 min, and incubated with goat anti-rabbit Ig diluted 1:2000 in normal sheep serum for 30 min at room temperature. Slides were washed three times in PBS for 10 min, and incubated with the DAB (diaminobenzidine tetrahydrochloride) substrate (5 mg DAB in 20 ml 0.03% H_2O_2 in PBS) for 10 min at room temperature in the dark. Slides were washed three times in PBS, counterstained in 0.15% methylene blue for 10 min, washed twice in tap water,

dehydrated by a series of dips in 70%, 95%, and 100% ethanol, dipped in xylol twice, and mounted with Permount.

Fish culture

While optimum values for most culture variables have not been determined for northern pike, our culture methods were based on known requirements of the species and empirical data (Westers, 1986; Coolwater Fish Culture Workshop Minutes, 1988, 1989, 1990). We set conservative values for maximum density index, minimum dissolved oxygen tension and maximum ammonia concentrations by using recommendations for rainbow trout *(Oncorhynchus mykiss),* a species requiring relatively high water quality (Meade, 1985; Piper et al., 1982; Soderberg, 1982).

Incubation and hatching. Microinjected and control embryos were separately incubated in divided compartments of a flow-through vertical tray incubator. Embryos were incubated at 10°C until hatching (approximately 9 days). Immediately before hatching, embryos were placed in plastic pans in which the water was allowed to warm to room temperature, thereby reducing variability in fry size by synchronizing hatching of all embryos within an hour.

Fry rearing. Microinjected and control fry were separately reared in 210-l circular tanks at similar densities, ranging from 10 fry/l at hatching to less than 1 fry/l (or 15 g/l) at 2 months of age. Water temperature and flows were kept constant at 21°C and one exchange per hour, respectively (Westers, 1986). After fry swim-up (approximately 3 days), automatic feeders delivered brine shrimp at 5-min intervals during 24 h of light. After 10 days, fry were converted to BioDiet pelleted feed (size #2; BioProducts Inc.), which was delivered by automatic Louden trough feeders (North Star Co.) at frequent intervals during light hours. Dual feeding of brine shrimp and BioDiet usually lasted 3 days.

Rearing after 2 months of age. Water temperatures, flow rates, and delivery of pelleted feed were as described for fry rearing. Densities ranged from 15 g/l when average fish length was 12 cm to 95 g/l when average fish length was 30 cm. Photoperiod was 14 h light:10 h dark and feed type Glencoe Trout Grower pellets (International Multifoods). When individual weights averaged 15 g, microinjected and control fish of an experiment were tagged intraperitoneally with passive integrated transponders (Biosonics Corp.; Prentice et al., 1987) which permit individual identification. All microinjected and control individuals in a given growth experiment were then transferred to a common rearing tank.

TABLE 1

Percent hatch and numbers of fish retained for growth experiments

Expt.	Construct	Percent hatch		No. males analyzed		No. females analyzed	
		Injected	Control	Injected	Control	Injected	Control
A	pRSV/bGH	62	58	22	7	24	9
B	pRSV/bGH	63	78	38	17	98	26
C	FV-2/csGH	66	75	61	7	67	24
	Totals	64[a]	70[a]	121	31	189	59

[a]Average value.

Growth analysis

A randomly chosen subset of the molecularly analyzed fish was compared to controls in three growth experiments (Table 1). Individual weights (to the nearest 0.1 g) and lengths (to the nearest 1.0 mm) were recorded on a monthly basis. As fish gained weight and space became limiting, numbers of fish in each experiment were reduced to maintain optimum rearing conditions. To cull fish, a computer-generated random number was assigned to each individual within the control or microinjected group in a common tank, the numbers were sorted and the appropriate numbers of fish were eliminated from the top of the list for the group.

Because females are usually larger than males and skewed sex ratios are known to exist in populations of northern pike (Hassler, 1969; Scott and Crossman, 1985), growth data were analyzed by sex. At 1 year of age, fish were sexed based on the shape of the urogenital opening (Demchenko, 1963; Casselman, 1974). Our ability to determine sex in this manner was verified by dissection of 100 fish. Weight differences between treatments of the same sex were analyzed by *t*-tests at approximately 6, 9, and 12 months of age. *F*-statistics were calculated to test the equality of variances and determine whether pooled or separate variance *t*-tests applied (Zar, 1984).

RESULTS

By microinjection into the germinal disc of one-cell northern pike embryos, we obtained successful transfer and genomic integration of cDNA for either: bovine growth hormone linked to the Rous sarcoma virus long terminal repeat (pRSV/bGH; Fig. 2); or chinook salmon growth hormone linked to the carp β-actin promoter (FV-2/csGH; Fig. 1). For some fish microinjected with pRSV/bGH, we detected expression of bGH in blood serum and growth enhancement under controlled growth trials. In earlier studies (Liu et al., 1990a),

we demonstrated expression of transgenes in fish under the transcriptional control of the carp β-actin promoter.

Production of transgenic northern pike

For the three growth experiments (Table 1), a total of 14 000 one-cell northern pike embryos was microinjected. Hatching rates for both control (58–78%) and microinjected (62–66%) groups were normal for artificial incubation of this species (Westers, 1986). Of the surviving microinjected fish, 440 were randomly chosen for growth analyses (Table 2). In addition to these growth experiments, another 10 000 embryos were microinjected, of which 958 juvenile fish were molecularly analyzed (Table 2).

DNA analysis

As shown in Fig. 3A, uncut genomic DNA from 6-day-old pRSV/bGH microinjected embryos appeared to be in two forms: linear copies (3.9 Kb) and high molecular weight DNA. The latter form, which represents the majority of the DNA, migrated as two separate bands: a band ($\sim$23 Kb) representing large concatamers of the injected DNA; and a band larger than 40 Kb that migrated with cellular DNA and represents integrated transgenic DNA. In the lane for embryonic DNA digested with *Bam*HI several prominent bands exist: (1) the 3.9 Kb band represents linear copies of pRSV/bGH derived from digestion of both integrated and concatamer forms as well as the free linear copies present in the uncut lane; (2) the two faint high molecular weight bands most likely represent trimers and larger concatamers that have lost the *Bam*HI site during concatamerization; (3) the unexplainable 2.2 Kb band was found in all 6-day-old microinjected embryos analyzed but not in control embryos (data not shown). The exact nature of the band is unknown at this point, but probably represents a common deletion product of the microinjected DNA.

TABLE 2

Numbers of presumptive juvenile transgenic fish molecularly analyzed and numbers in which transgene DNA or protein product (bGH) was detected

Expt.[a]	Fish analyzed	DNA fin positive	Serum bGH
A	52	6 (11%)	nd
B	260	29 (11%)	7 (3%)
C	128	9 (7%)	nd
D[b]	958	44 (5%)	29 (3%)
Total	1398	88 (6%)	36 (3%)[c]

[a]DNA constructs used are described in Table 1.
[b]These fish were microinjected with pRSV/bGH but were not part of a growth experiment.
[c]Represents 3% of total for experiments B and D (36/1218).
nd = not done.

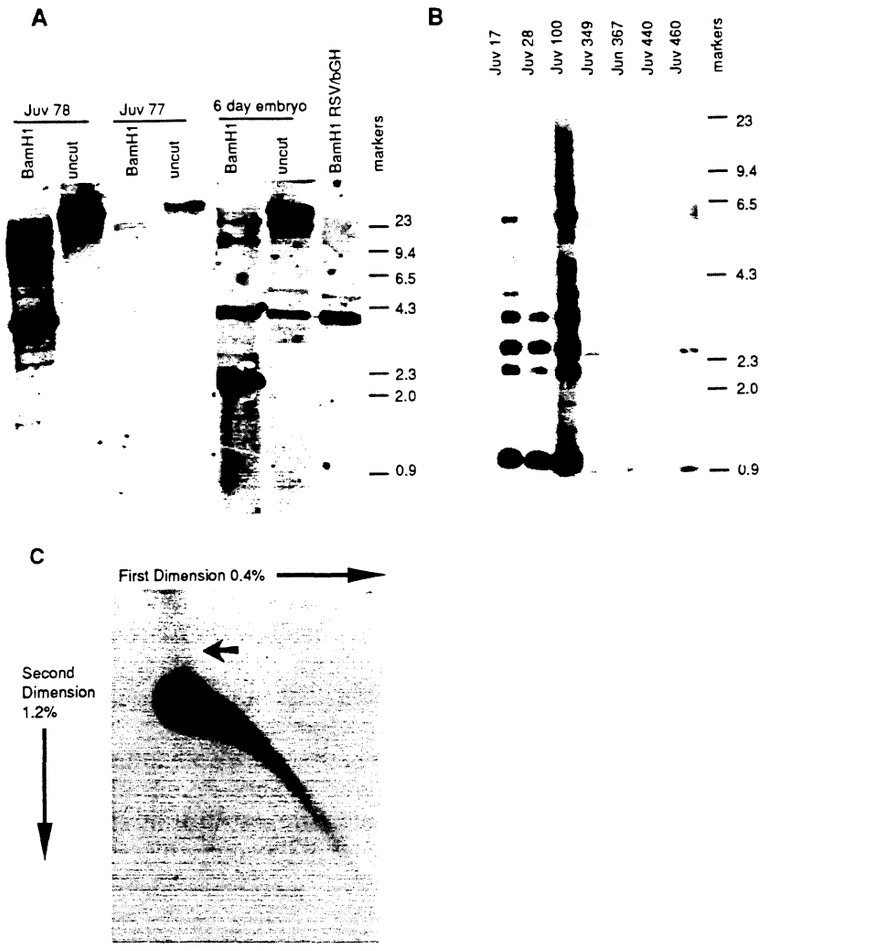


Fig. 3. Southern blot hybridization analysis of DNA from selected northern pike transgenic for pRSV/bGH. (A) 10 μg of either *Bam*HI digested or uncleaved DNA from embryos was electrophoresed and transferred to nylon membranes, then hybridized to a [^{32}P]-labelled probe made from a *Bam*HI/*Hind*III (0.83 Kb) fragment of pRSV/bGH containing the coding region of bGH. 20 pg of linearized pRSV/bGH cleaved with *Bam*HI is included as a control. Lambda DNA cleaved with *Hin*dIII was used for size markers (fragment sizes given in Kb). (B) 5 μg of DNA from juvenile (Juv) northern pike fins were cleaved with *Eco*RI, electrophoresed, transferred, then hybridized to a [^{32}P] labeled probe made from the entire pRSV/bGH plasmid. Expected band sizes corresponding to *Eco*RI cleaved pRSV/bGH are 0.96 and 2.9 Kb. *Hin*dIII cleaved lambda phage DNA served as size markers (fragment sizes given in Kb). The bands at 2.5 and 2.1 Kb represent vector sequences from a co-microinjected pRSV/CAT plasmid (Hallerman et al., 1990) included in some Juv fish. (C) Two-dimensional gel electrophoresis analysis of uncut genomic DNA from Juv17. Arrow indicates the expected location of episomal and concatamerized forms of DNA if they were present.

Two-month-old juvenile fish were sufficiently large to allow removal of fin tissue without affecting survival. Therefore, to determine which of the surviving microinjected fish were transgenic, DNA was extracted from fin clips and screened for the presence of transgenic DNA by Southern blot hybridization. As shown in Table 2, 5–11% of microinjected fish in experiments A–D and 6% (88) of all surviving juvenile fish contained detectable levels of transgenic DNA within their fin tissue. Unlike embryonic DNA, however, the transgenic DNA in juvenile fish appeared to be only in an integrated form. For example, in Fig. 3A, the pRSV/bGH transgenic sequences in the uncut Juv77 and Juv78 lanes only migrated as high molecular weight DNA. Furthermore, two-dimensional Southern blot hybridization analysis indicated that the transgenic DNA had probably been integrated into the genomes of juvenile fish (Fig. 3C). Transgenic DNA in the form of episomes or large supercoiled concatamers have been observed by other researchers; however, such forms would migrate above (see arrow Fig. 3C) the main bulk of the DNA. The possibility remains that some transgenic DNA is in a very long linear form, although such DNA generally does not have a long half-life in developing organisms. Additionally, dominant bands from 70% of pRSV/bGH transgenic fish DNA's, when analyzed by Southern hybridization, migrated at the expected band sizes: *Bam*HI linearizes pRSV/bGH and produces a 3.9 Kb fragment; whereas *Eco*RI digestion gives bands of 0.96 and 2.9 Kb. For example, DNA of Juv78 digested with *Bam*HI produced a dominant band at 3.9 Kb representing linear pRSV/bGH as well as many putative junction fragments (Fig. 3A). In Fig. 3B, all juvenile fish shown were digested with *Eco*RI and yielded the expected bands at 0.96 and 2.9 Kb. Putative junction fragments are also evident. However, other samples, including Juv77 (Fig. 3A) gave bands upon restriction digestion that were of unexpected sizes, suggesting transgene rearrangement. Transgene copy number per fish ranged from less than one to hundreds of copies.

bGH assays

Because non-lethal removal of blood from 2-month-old fish was also possible, blood serum from these same fish was analyzed for the presence of circulating bovine growth hormone (Table 2). Radioimmunoassays detected bGH hormone in the serum of 3% (36) of the tested fish. The majority of these positive fish had levels below 4 ng/ml of circulating bGH hormone within their serum, although nine fish had levels above 10 ng/ml with the highest being 37 ng/ml. Interestingly, 34 of the 36 fish had no detectable transgenic DNA within their fins, implying a high degree of mosaicism within the transgenic fish.

Lack of any positive results with blood serum from control fish indicated that our bGH radioimmunoassay did not cross-react with native northern pike growth hormone. Additional evidence supporting this conclusion came

Fig. 4. Thin slice immunohistochemical analysis of northern pike fins employing a polyclonal rabbit anti-bGH antibody. Panel A shows fin tissue from a control fish. Panel B shows fin tissue from a fish microinjected with pRSV/bGH whose transgene expression is limited to undifferentiated mesenchymal tissue, appearing as dark areas (especially prevalent within the chondrocytes). Abbreviations for tissue types are: (ch) chondrocytes, (epi) epithelial tissue, (ray) fin ray, and (um) undifferentiated mesenchymal tissue.

TABLE 3

The extent of tissue DNA mosaicism in a random sample of northern pike microinjected with pRSV/bGH

Fish no.	Brain	Gonad	Kidney	Liver	Muscle	Fin
1	–	–	+	+	–	–
14	–	–	+	+	–	–
16	+	–	+	+	+	+
19	–	–	+	+	+	+
20	–	–	+	–	–	–
21	–	–	–	–	+	+
24	–	–	+	+	+	–
26	–	–	+	–	–	–
27	+	+	+	+	+	+
29	+	+	+	–	–	–
31	–	–	+	–	–	–
36	–	–	–	–	+	+
Total[a]	3/12	2/12	10/12	6/1	6/12	5/12
% of positive fish	25	16	83	50	50	41
% of all analyzed fish	7	5	25	15	15	12

[a]12/40 fish were positive in one or more tissues, or 30% overall.

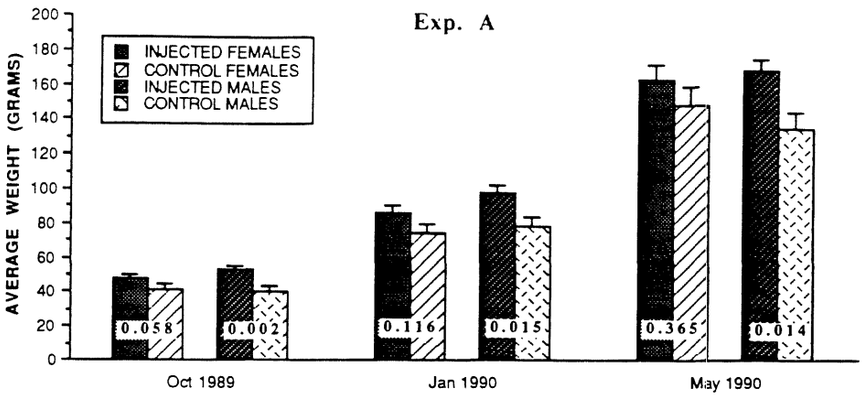
from tests with a chinook salmon GH assay. This assay, which cross-reacted with northern pike blood, showed no cross-reactivity with purified bGH (Brian McKeown, Dept. of Biological Sciences, Simon Fraser University, Burnaby, B.C., personal communication).

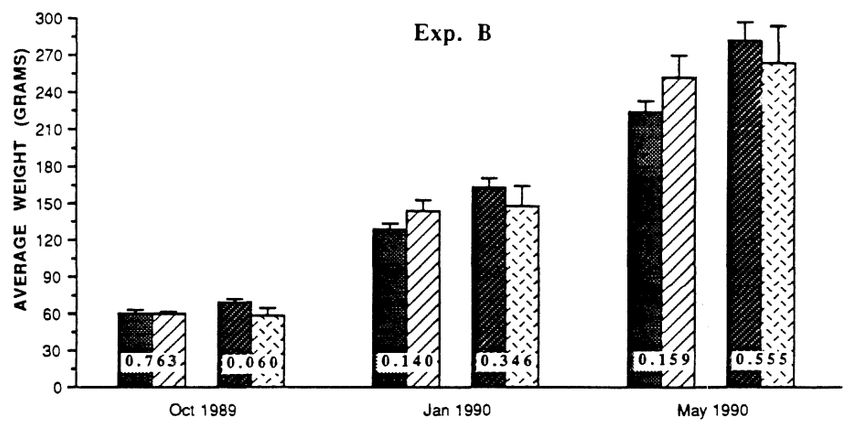
Immunohistochemical detection of bGH in fins

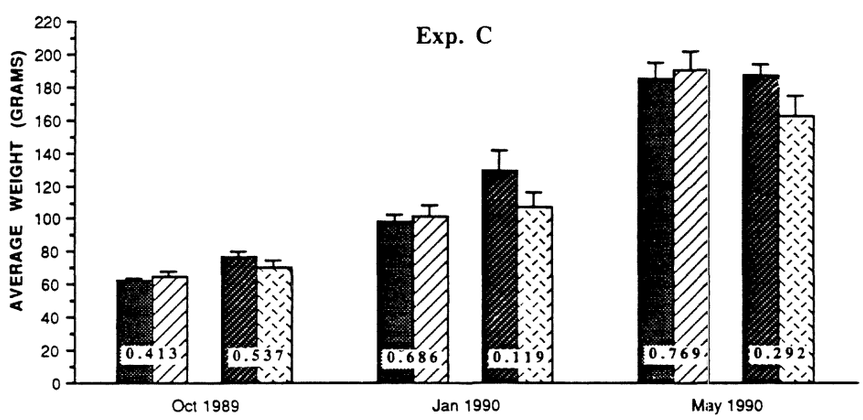
Two fish, with bGH in their serum but no detectable transgene in their fins, expressed bGH protein within their fins, as detected by a thin slice immunohistochemistry assay employing a polyclonal rabbit anti-bGH antibody (Fig. 4). Expression was restricted to a thin layer of mesodermal tissue within the fin, and was especially prevalent within the chondrocytes. The identical expression pattern was found in similar thin slices from several fish that were positive for both transgene DNA in fin tissue and for bGH in serum. At this time, we do not know whether transgene DNA is present in a percentage of all the various types of fin cells, or only in those cells with observed expression of the hormone.

Detection of mosaicism

Because transgenic fish appeared to be highly mosaic, we analyzed six tissues (brain, gonad, kidney, liver, muscle, and fin) from 40 randomly selected


Exp. A
INJECTED FEMALES
CONTROL FEMALES
INJECTED MALES
CONTROL MALES
AVERAGE WEIGHT (GRAMS)
200
180
160
140
120
100
80
60
40
20
0
0.058
0.002
0.116
0.015
0.365
0.014
Oct 1989
Jan 1990
May 1990


Exp. B
AVERAGE WEIGHT (GRAMS)
300
270
240
210
180
150
120
90
60
30
0
0.763
0.060
0.140
0.346
0.159
0.555
Oct 1989
Jan 1990
May 1990


Exp. C
AVERAGE WEIGHT (GRAMS)
220
200
180
160
140
120
100
80
60
40
20
0
0.413
0.537
0.686
0.119
0.769
0.292
Oct 1989
Jan 1990
May 1990

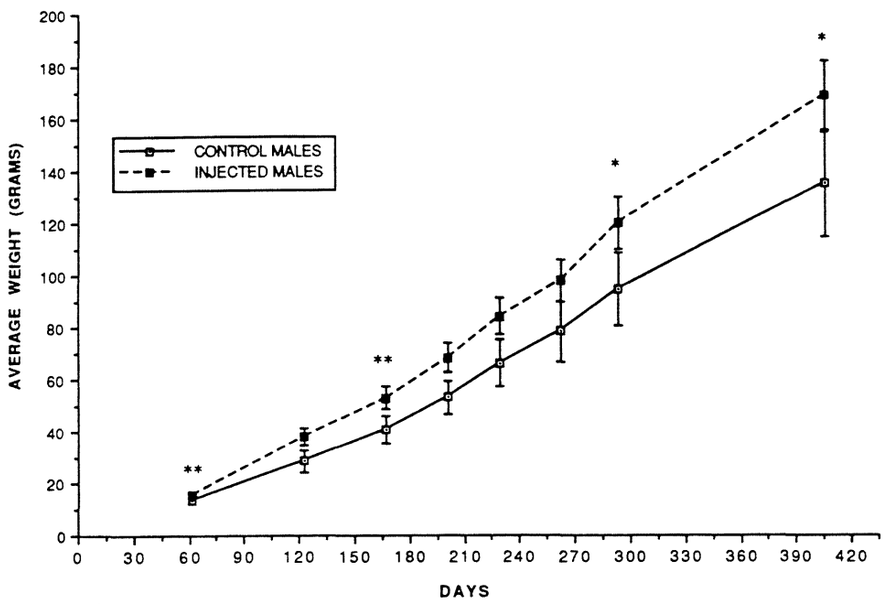


Fig. 6. Average weight (g) of males in Experiment A (pRSV/bGH) as a function of days since hatching. Asterisks denote *t*-test comparisons ($^{*}P<0.05$ and $^{**}P<0.01$). Error bars represent twice the standard errors of means.

fish for the presence of transgenic DNA by Southern hybridization (Table 3). Thirty percent of the fish from this sample contained detectable transgenic DNA in at least one or more tissues, with the kidney being the most prevalent (83% of positives). Only one fish (No. 27) was found to have DNA present within all examined tissues. Interestingly, transgene DNA was only detectable within fin tissues of 41% of the positive fish (or 12% of all examined fish), indicating our non-lethal screening procedure did not identify all transgenic individuals.

Growth analyses

Average weight of microinjected fish was greater than that of control fish of the same sex in four out of six groups (Fig. 5): females and males of experiment A, males of experiment B, and males of experiment C. At this point in

Fig. 5. Average weight (g), by sex, of microinjected and control fish for each growth experiment for three different time periods. DNA constructs used in each experiment are described in Table 1. The age of the fish, in days since hatching, were (for Expt. A–C): October 1989, 167–182 days; January 1990, 242–262 days; May 1990, 386–406 days. The *P*-values of *t*-test comparisons by sex for microinjected and control fish are superimposed on corresponding bars.

our ongoing study, however, microinjected males in experiment A are the only experimental group whose weights were significantly different ($P<0.05$) from those of controls (Fig. 5). Length data (not shown) yielded the same trends as weight data in all experiments. Weights of microinjected and control males in experiment A steadily diverged over time (Fig. 6), 280 a pattern also noted in females of experiment A and males of experiment C (data not shown). Similar patterns of divergence in growth have been observed in studies involving injections of bGH into fish (Weatherly and Gill, 1987a; Schulte et al., 1989). Fish positive for either DNA within their fins or bGH expression within their blood were also compared as a group to controls by *t*-tests. Although they followed the same trends as seen for comparisons involving entire microinjected groups, weight differences from these group comparisons were insignificant (data not shown).

In every experiment the largest individuals of either sex were from the microinjected group, totalling 43 fish for the three experiments. Transgene DNA was detected in fin tissue of only three of these 43 fish. If the remainder of these fish are larger due to proper expression of transgenes, they must bear integrated and expressed transgenes in tissues undetectable by nonlethal methods.

DISCUSSION

Using standard techniques of microinjection into one-cell embryos, we obtained integration rates in juvenile fish (5–11% and 6% overall in fin tissue) comparable to those reported for common carp, *C. carpio* (5.5% in fin tissue; Zhang et al., 1990), tilapia, *Oreochromis niloticus* (6% in whole fish samples; Brem et al., 1988), and zebrafish, *Brachydanio rerio* (5% in fin tissue; Stuart et al., 1988). A higher integration rate (20% in whole fish samples) reported for juvenile channel catfish, *Ictalurus punctatus* (Dunham et al., 1987), was based on a sample size of 2 transgenic fish out of 10 microinjected embryos.

Our detection of circulating bGH in 3% of the analyzed microinjected fish indicated transgene expression but involved small numbers of individuals (≤ 4 of each sex) within a growth experiment. Because this reduced statistical power, we did not conduct *t*-test comparisons of their growth to that of controls. We do not know if the minimum effective levels of bGH for growth enhancement are below or above the detection threshold of our assay, which was approximately 1 ng/ml. Individual levels of circulating bGH detected by our radioimmunoassays ranged from 2.0 ng/ml to 37.0 ng/ml. Reports of basal levels of endogenous growth hormone for fish include 7.2–58.4 ng/ml for chum salmon, *Oncorhynchus keta* (Bolton et al., 1986), 10–60 ng/ml for goldfish, *Carassius auratus* (Marchant and Peter, 1986), and 10–70 ng/ml for white sucker, *Catostomus commersoni* (Stacey et al., 1984). We also do not know how levels of hormone expression are developmentally regulated.

Given these unknowns, it is possible to miss detection of physiologically active levels of bGH in transgenic individuals showing growth enhancement.

Comparisons among data for integrated transgenes in fin DNA, serum bGH assays, and immunohistochemical detection of bGH in fin tissue indicated a high incidence of mosaic individuals in our founder generation of live transgenic fish. This was confirmed by detection of transgene DNA in at least one of six examined tissues for 30% of 40 sacrificed fish. This is consistent with results of a previous study showing the distribution and expression of a marker transgene in goldfish (Hallerman et al., 1990). Stuart et al. (1988) also detected mosaicism in the founder generation of zebrafish based on the incidence of germ-line transmission and on the distribution of transgenes in eight tissues of one female. Patterns of germ-line transmission for the founder generation of common carp also indicated mosaicism (Zhang et al., 1990), although tissue comparisons of transgene integration and expression within individuals were not reported.

In this study we detected bGH expression in mesodermal tissue of fins and, in a previous report, we localized the prevalence of RSV-LTR-driven expression to mesodermal tissues in transgenic goldfish (Hallerman et al., 1990). Both reports parallel the incidence of tumorigenesis in such tissues following RSV virus infection into chickens and mice (Svet-Modalvsky, 1958; Purchase and Burmester, 1978). The molecular basis of this tissue tropism is not completely understood, but is presumed to be due to interactions between tissue specific transcription factors and the RSV-LTR. These same factors may therefore occur within fish, suggesting their high degree of conservation (Liu et al., 1990b), as well as indicating the value of transgenic fish as a model system for elucidating possible mechanisms in transcriptional control.

Our growth results represent a systematic evaluation of performance of founder generation transgenic fish under controlled rearing conditions. While the largest individuals in each experiment had been microinjected, significant growth enhancement ($P<0.05$) was observed only for microinjected males in one experiment (Figs. 5 and 6). Detection of statistically significant growth enhancement was influenced by three factors: presence of a mixture of genotypes in the founder generation of presumptive transgenic fish; variation in biological activity of the transgene product; and behavioral interactions.

Each microinjected group analyzed for growth performance was handled as one treatment for statistical analysis (F. Martin, Dept. of Applied Statistics, University of Minnesota, St. Paul, personal communication). However, each group actually consisted of several genotypes to which a given live individual could not be accurately assigned. Possible genotypes include: nontransgenic fish (probably > 50% based on results shown in Table 2 and 3); mosaics which lack proper transgene expression, thus performing like nontransgenics; mosaics which express the transgene in relevant tissues (many of which will be undetectable without sacrificing the fish); and completely transgenic individ-

uals which contain transgene DNA within all their cells and express it in a usable manner. The latter is the desired genotype but it probably occurs in only a small fraction of founder individuals using the current technique of microinjection at the one-cell stage of embryos.

Transgene expression leading to proper physiological activity is believed to require secretion of growth hormone into the bloodstream, whereby it is transported to target tissues and binds to cell surface receptors (Weatherly and Gill, 1987b). Therefore, if some founder generation individuals had expression of transgenes in non-target tissues without secretion into the bloodstream, they would not be expected to display growth enhancement. Lack of expression in some transgenic individuals could result from partial enzymatic degradation of the construct prior to incorporation (Maclean et al., 1987) or from rearrangement of the construct. Our Southern hybridization data indicated rearrangement of integrated transgenes in some founder generation individuals.

At this time, we can only speculate about the impact of behavioral interactions within common rearing tanks on observed growth differences in our experiments. Aggressive behavior of a few individuals has been shown to suppress growth of other fish in common tanks (Magnuson, 1962; Weatherly and Gill, 1987b). Additional experiments are needed to test specific hypotheses about the impact of behavioral interactions on growth differences between fish bearing growth promoting transgenes and controls.

As our fish reach sexual maturity, we are breeding them to produce populations of non-mosaic transgenic fish by germ-line transmission and are screening for progeny containing integrated sequences that are not rearranged. Elimination of mosaic genotypes will allow correct identification of transgenic individuals by non-lethal means, thus reducing ambiguities in assays for GH expression and assessment of growth performance. Current plans are to compare growth of transgenic progeny to controls in common and separate rearing tanks. This will permit partitioning of behavioral effects from direct physiological effects of growth hormone expressed by transgenes.

ACKNOWLEDGEMENTS

The authors would like to thank the Minnesota Department of Natural Resources for the provision of fish gametes, Dr. Choy Hew (Department of Clinical Biochemistry, University of Toronto) for supplying the chinook salmon growth hormone cDNA, and Dr. Ronald McGlennen, Dr. Brian McKeown, Dr. John Wheaton, Kris Carlson, Kurt LaBresh, Sung Joo Yoon, Zuoyan Zhu, Long He, Jay Maher, Mark Dalton and Mike Voss for their technical assistance and advice. The University of Minnesota Veterinary Pathobiology lab prepared slides of fin tissue sections. The anti-bGH antibodies were a gift from the National Hormone and Pituitary Program (University

of Maryland School of Medicine). Support for this work came in part from the Minnesota Agricultural Experiment Station, the Minnesota Sea Grant College Program supported by the NOAA Office of Sea Grant, Department of Commerce under Grant Nos. NOAA-86AA-D-SG112, Project Nos. RA/3, RA/5, RA/6 and NOAA-90AA-SG149, Project Nos. RA/5, RA/6, RA/8, J. R. 267, the Legislative Commission on Minnesota Resources, The Blandin Foundation, and the Greater Minnesota Corporation. This is article 18 723 of the Minnesota Agricultural Experiment Station Scientific Journal Article Series.

REFERENCES

Adelman, I.R., 1977. Effect of bovine growth hormone on growth of carp *(Cyprinus carpio)* and the influences of temperature and photoperiod. J. Fish. Res. Board Can., 34: 509–515.

Agellon, L.B. and Chen, T.T., 1986. Rainbow trout growth hormone: molecular cloning of cDNA and expression in *Escherichia coli*. DNA, 5(6): 463–471.

Bolton, J.P., Takahashi, A., Kawauchi, H., Kubota, J. and Hirano, T., 1986. Development and validation of a salmon growth hormone radioimmunassay. Gen. Comp. Endocrinol., 62: 230–238.

Brem, G., Brenig, G., Hörstgen-Schwark, G. and Winnacker, E.L., 1988. Gene transfer in tilapia *(Oreochromis niloticus)*. Aquaculture, 68: 209–219.

Casselman, J.M., 1974. External sex determination of northern pike, *Esox lucius* Linnaeus. Trans. Am. Fish. Soc., 103(2): 343–347.

Chourrout, D., Guyomard, R. and Houdebine, L., 1986. High efficiency gene transfer in rainbow trout (*Salmo gairdneri* Rich.) by microinjection into egg cytoplasm. Aquaculture, 51: 143–150.

Coolwater Fish Culture Workshop Minutes, 1988. Aberdeen, South Dakota, 4–7 January 1988 (unpubl.).

Coolwater Fish Culture Workshop Minutes, 1989. South Bend, Indiana, 10–11 January 1989 (unpubl.).

Coolwater Fish Culture Workshop Minutes, 1990. Bozeman, Montana, 8–10 January 1990 (unpubl.).

Demchenko, I.F., 1963. On sexual differences in the pike *(Esox lucius)*. Vopr. Ikhtiol., 3: 190–193 (in Russian).

Dunham, R., Eash, J., Askins, J. and Townes, T.M., 1987. Transfer of the metallothionein–human growth hormone fusion gene into channel catfish. Trans. Am. Fish. Soc., 116: 87–91.

Guise, K.S., Faras, A.J., Hackett, P.B. and Kapuscinski, A.R., 1990. Gene transfer in fish. In: N.I. First and F.S. Haseltine (Editors), Transgenic Animals, Proceedings of the Symposium on Transgenic Technology in Medicine and Agriculture. Butterworth-Heinemann, Boston, 358 pp.

Hallerman, E.M., Schneider, J.F., Gross, M., Liu, Z., Yoon, L., He, L., Hackett, P.B. and Kapuscinski, A.R., 1990. Gene expression promoted by the RSV long terminal repeat element in transgenic goldfish. Anim. Biotech., 1(1): 79–93.

Hassler, T.J., 1969. Biology of the northern pike in Oahe Reservoir, 1959 through 1965. U.S. Bureau of Sport Fisheries and Wildlife, Technical Paper, 29: 1–13.

Hew, C.L., Trinh, K.Y., Du, S.J. and Song, S., 1989. Molecular cloning and expression of salmon pituitary hormones. Fish Physiol., Biochem., 7: 375–380.

Kapuscinski, A.R. and Hallerman, E.M., 1990. Transgenic fish and public policy. I. Anticipating environmental impacts of transgenic fish. Fisheries, 15(1): 2–11.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B., 1990a. Functional analysis of elements affecting expression of the β-actin gene of carp. Mol. Cell. Biol., 10(7): 3432–3440.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B., 1990b. Development of expression vectors for transgenic fish. Bio/Technology, 8: 1268–1272.

Maclean, N., Penman, D. and Zhu, Z., 1987. Introduction of novel genes into fish. Bio/Technology, 5: 257–261.

Magnuson, J.H., 1962. An analysis of aggressive behaviour, growth, and competition for food and space in medaka *(Oryzias latipes)* (Pisces, Cyprinodontidae). Can. J. Zool., 40: 313–362.

Manias, D.A., Ostrow, R.S., McGlennen, R.C., Estensen, R.D. and Faras, A.J., 1989. Characterization of integrated human papillomavirus type 11 DNA in primary and metastatic tumors from a renal transplant recipient. Cancer Res., 49: 2514–2519.

Marchant, T.A. and Peter, R.E., 1986. Seasonal variations in body growth rates and circulating levels of growth hormone in the goldfish, *Carassius auratus*. J. Exp. Zool., 237: 231–239.

Meade, J.W., 1985. Allowable ammonia for fish culture. Prog. Fish-Cult., 47(3): 135–145.

Palmiter, R.D., Brinster, R.L., Manner, R.E., Trumbauer, M.E., Rosenfeld, M.C., Birnberg, N.C. and Evans, R.M., 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein growth hormone fusion genes. Nature, 300: 611–615.

Piper, R.G., McElwain, I.B., Orme, L.E., McCraren, J.P., Fowler, L.G. and Leonard, J.R., 1982. Fish Hatchery Management. U.S. Dept. of the Interior, Fish and Wildlife Service, Washington, DC, 517 pp.

Prentice, E.F., Flagg, T.A. and McCutcheon, S., 1987. A study to determine the biological feasibility of a new fish tagging system, 1986–87. Report to Bonneville Power Admin., Project 83-319, Seattle. 113 pp.

Purchase, H.G. and Burmester, B.R., 1978. Leukosis/sarcoma group. In: M.S. Hofstad, B.W. Calnek, C.F. Helmboldt, W.M. Reid and H.W. Yoder (Editors), Diseases of Poultry. Iowa State Univ. Press, Ames, 949 pp.

Pursell, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campell, R.G., Palmiter, R.D., Brinster, R.L. and Hammer, R.E., 1989. Genetic engineering of livestock. Science, 244: 1281–1288.

Rokkones, E., Alestrom, P., Skervold, H. and Gautvik, K.M., 1989. Microinjection and expression of a mouse metallothionein human growth hormone fusion gene in fertilized salmonid eggs. J. Comp. Physiol. B., 158: 751–758.

Schulte, P.M., Down, N.E., Donaldson, E.M. and Souza, L.M., 1989. Experimental administration of recombinant bovine growth hormone to juvenile rainbow trout *(Salmo gairdneri)* by injection or by immersion. Aquaculture, 76: 145–156.

Scott, W.B. and Crossman, E.J., 1985. Freshwater Fishes of Canada. Fisheries Research Board of Canada Bulletin 184, Ottawa, 966 pp.

Soderberg, R.W., 1982. Aeration of water supplies for fish culture in flowing waters. Prog. Fish-Cult., 44: 89–93.

Stacey, N.E., MacKenzie, D.S., Marchant, T.A., Kyle, A.L. and Peter, R.E., 1984. Endocrine changes during natural spawning in the white sucker, *Catostomus commersoni*. I. Gonadotropin, growth hormone, and thyroid hormones. Gen. Comp. Endocrinol., 56: 333–348.

Stickney, R., 1990. A global overview of aquaculture production. Food Rev. Int., 6: 299–315.

Stuart, G.W., McMurray, J.V. and Westerfield, M., 1988. Replication, integration, and stable germ line transmission of foreign sequences injected into early zebrafish embryos. Development, 103: 403–412.

Svet-Modalvsky, G.J., 1958. Sarcoma in albino rats treated during the embryonic stage with Rous virus. Nature, 182: 1452–1453.

Weatherly, A.H. and Gill, H.S., 1987a. Growth increases produced by bovine growth hormone in grass pickerel, *Esox americanus vermiculatus* (LeSueur), and the underlying dynamics of muscle fiber growth. Aquaculture, 65: 55–66.

Weatherly, A.H. and Gill, H.S., 1987b. The Biology of Fish Growth. Academic Press, London, 443 pp.

Westers, H., 1986. Northern pike and muskellunge. In: R. Stickney (Editor), Culture of Non-salmonid Freshwater Fishes. CRC Press, Boca Raton, FL, 201 pp.

Wheaton, J.E., Al-Raheem, S.N., Massri, Y.G. and Marcek, J.M., 1986. Twenty-four hour growth hormone profiles in Angus steers. J. Anim. Sci., 62: 1267–1272.

Yoon, S.J., Hallerman, E.M., Gross, M.L., Liu, Z., Schneider, J.F., Faras, A.J., Hackett, P.B., Kapuscinski, A.R. and Guise, K.S., 1990. Transfer of the gene for neomycin resistance into goldfish, *Carassius auratus*. Aquaculture, 85: 21–23.

Zar, J.H., 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ, 718 pp.

Zhang, P., Hayat, M., Joyce, C., Gonzalez-Villaseñor, L.I., Lin, C.M., Dunham, R.A., Chen, T.T. and Powers, D.A., 1990. Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio* (Linnaeus). Mol. Reprod. Dev., 25: 3–13.,

Zhu, Z., Li, G., He, L. and Chen, S., 1985. Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). Z. Angew. Ichthyol., 1: 31–34.

Zhu, Z., Xu, K., Li, G., Xie, Y. and He, L., 1986. Biological effects of human growth hormone gene microinjected into the fertilized eggs of loach *Misgurnus anguillicaudatus*. Kexue Tongbao Acad. Sin., 31(14): 988–990.

Molecular Marine Biology and Biotechnology (1992) 1(2). 106–112

Molecular cloning and sequence analysis of the cDNA for northern pike (Esox lucius) growth hormone

John F. Schneider, Steven H. Myster, Perry B. Hackett, Kevin S. Guise, and Anthony J. Faras*
The Institute of Human Genetics, the Departments of Genetics and Cell Biology, Animal Sciences, and Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, U.S.A.

Abstract

The complete nucleotide sequence of the northern pike (*Esox lucius*) cDNA for pregrowth hormone was determined from clones derived from a pituitary gland cDNA library. Seventeen cDNA clones were isolated from a single mRNA species. A cDNA of 1,227 nucleotides was sequenced and found to encode a polypeptide of 209 amino acid residues, which included a putative signal sequence of 22 amino acid residues. Sequence comparison of the northern pike growth hormone gene to other known growth hormone genes revealed similarities closest to other members of the superorder *Protacanthopterygii*, which includes the *Salmonidae* family (i.e., salmon and trout).

Introduction

Growth hormone (GH) is a single-chain polypeptide of approximately 22 Kd produced by the somatotrophs of the anterior pituitary gland. The mature hormone is processed from a precursor by removal of a short signal peptide. GH, together with prolactin (PRL) and chorionic somatomammoptropin (CS, placental lactogen), form a family of polypeptide hormones related by function and sequence similarity (Miller and Eberhardt, 1983). They are believed to have evolved from the same ancestral gene (Niall et al., 1971). In fish, GH and PRL are important hormones in the control of somatic growth and differentiation, osmoregulation, as well as other less defined biological functions.

* Correspondence should be sent to this author.

The recent application of biotechnology in aquaculture has increased the importance of isolating and characterizing piscine GH genes. The characterization of piscine GH genes will facilitate an understanding of their regulation and roles in growth, differentiation, and osmoregulation, as well as the evolutionary relationships between piscine and other species. To date, GH complementary DNAs (cDNAs) have been isolated from the following piscine species: chum salmon (Sekine et al., 1985), two from rainbow trout (Agellon and Chen 1986; Rentier-Delrue et al., 1989), coho salmon (Nicoll et al., 1987), bluefin tuna (Sato et al., 1988), red sea bream (Momota et al., 1988), flounder (Mori et al., 1989), yellowtail (Watahika et al., 1988), atlantic salmon (Lorens et al., 1989), chinook salmon (Hew et al., 1989), and carp (Chao et al., 1989). We describe the isolation and characterization of the northern pike GH and compare it to other fish growth hormones.

Results and Discussion

Nucleotide sequence of northern pike growth hormone cDNA

One hundred thirty-one positive clones were identified from a total of 1,800 colonies screened with the 5′ end of the rainbow trout GH cDNA. Inserts were analysed by gel electrophoresis from 21 prospective clones, and 18 were sequenced. No sequence differences were detected between any of these clones. One of the longest, pNPGH12, is reported herein. The complete nucleotide sequence for the northern pike GH cDNA is given in Figure 1. Excluding the poly(A) tail, the cDNA is 1,227 base pairs (bp) long and encodes a pre-GH of 209 amino acids. By comparison to other fish GHs, the northern pike GH contains a putative signal peptide of 22 amino acids in length and a mature hormone of 187 amino acids. The 5′ untranslated leader is 55 bp long, whereas the 3′ untranslated region is 544 bp in length. Two poly(A) addition signals (AATAAA) are included near the end of the 3′ region (1,069 and 1,205 bps, respectively).

```
                                                                                                                    bp  aa
  C TAA AGC ACA CAT TCA AGC TAT ACA AGT CCA TCC TCT GAC AGT TAG GAG AAG AAA ATG GGA                                  61
                                                                                                  MET Gly           -21

CAA GTG TTT CTG CTC ATG CCA GTC TTA CTG GTC GCT GGC TAT CTG AGT CTA GGT GCA GCA*ATG                                124
Gln Val Phe Leu Leu MET Pro Val Leu Leu Val Ala Gly Tyr Leu Ser Leu Gly Ala Ala*MET                                  1

GAG AAT CAG CGG CTG TTT AAC ATT GCT GTC AAC CGG GTG CAA CAT CTC CAC CTC CTG GCC CAG                                187
Glu Asn Gln Arg Leu Phe Asn Ile Ala Val Asn Arg Val Gln His Leu His Leu Leu Ala Gln                                 22

AAA ATG TTC AAC GAC TTC GAG GGC ACT CTG CTG CCT GAT GAA CGC AGA CAG TTG AAC AAG ATC                                250
Lys MET Phe Asn Asp Phe Glu Gly Thr Leu Leu Pro Asp Glu Arg Arg Gln Leu Asn Lys Ile                                 33

TTC CTC CTG GAC TTC TGT AAC TCC GAC TCC ATT GTG AGC CCC ATC GAC AAG CAC GAG ACT CAG                                313
Phe Leu Leu Asp Phe Cys Asn Ser Asp Ser Ile Val Ser Pro Ile Asp Lys His Glu Thr Gln                                 64

AAG AGT TCG GTC CTG AAG CTG CTC CAC ATT TCC TTC CGC CTG ATC GAG TCC TGG GAG TAC CCT                                376
Lys Ser Ser Val Leu Lys Leu Leu His Ile Ser Phe Arg Leu Ile Glu Ser Trp Glu Tyr Pro                                 85

AGC CAG ACG CTG ACC CAC ACC ATG TCC AAC AAC TTA AAC CAG AAC CAG ATG TCT GAG AAG CTC                                439
Ser Gln Thr Leu Thr His Thr MET Ser Asn Asn Leu Asn Gln Asn Gln MET Ser Glu Lys Leu                                106

AGC AAC CTC AAA GTG GGC ATC AAC CTG CTG ATC AAG GGC AAC CAG GAG GAT GTA CCA AGC CTG                                502
Ser Asn Leu Lys Val Gly Ile Asn Leu Leu Ile Lys Gly Asn Gln Glu Asp Val Pro Ser Leu                                127

GAT GAC AAC GAC TCT CAG CAG CTG CTC CCT TAT GGG AAC TAT TAC CAG AAC CTG GGA GAT AAC                                565
Asp Asp Asn Asp Ser Gln Gln Leu Leu Pro Tyr Gly Asn Tyr Tyr Gln Asn Leu Gly Asp Asn                                148

GAC AAC GTC AGA AGA AAC TAC GAG CTT CTG GCC TGC TTC AAA AAA GAC ATG CAC AAG GTT GAG                                628
Asp Asn Val Arg Arg Asn Tyr Glu Leu Leu Ala Cys Phe Lys Lys Asp MET His Lys Val Glu                                169

ACC TAC TTG ACG GTC GCC AAG TGC AGG AAG TCT CTG GAG GCC AAC TGC ACT CTG TAG GAT GGG                                691
Thr Tyr Leu Thr Val Ala Lys Cys Arg Lys Ser Leu Glu Ala Asn Cys Thr Leu                                            187

TCG GAG AGG CAG CCT GAT ACC ACT GGA CCA GTT TCG CAG GAA ATA GAT AGC ATC TCG TCC TGC                                754

ATG GAA AAC CAT TTT CAA TCC ATA TGA AAT GCT TTT CAG TGT AGT GGG TTC AGT CTA AAT CCA                                817

GCA ACC CGG CTC CAG GGG TTT TCA GGC ACC TGC ATT GTT CTC TGA AAT CTA CAA CAA CTT CAC                                880

TTA TTA TAT TCA CTT TAT TTC TCT GAG CTA TTA TTG ATT TGT GGA ATT CAT AGA TTA GTA CAT                                943

TCA TAG AAA CAT TTT TTG AAT GTT TTA ATT AAG ATA TCT GAT TCA AGG TGG TGC TGC AGT CAA                               1006

TGC ATA CAT TTA TTT TAG GCG TGG ATT CAC ACT GAC ATA AAA AAT AAC TAC TAA AAT GGG CAA                               1069

AAT AAA TGG TGG TCT CTG CAT AAG CGG CTT GAA GCT TTT GGG ATG TAC ACT GAT TAA TAA TCA                               1132

TGT CAT TTT CTC TAG GTT ATT TTC ATT CCA TTA CCC TAT TGT TTT AAT CTA TGT AGT AGT GCT                               1195

TCA TTT TTC AAT AAA GTC TGT TTG TTC TCT GC poly(A)n                                                               1227
```

Figure 1. The complete nucleotide sequence and deduced amino acid sequence of northern pike pregrowth hormone. *Asterisks* indicate the putative cleavage site between the signal peptide and the mature hormone. The polyadenylation site (AATAAA) and cysteine residues are underlined. Positive amino acid residue numbers correspond to the mature GH, whereas negative numbers refer to the signal peptide.

Amino acid sequence similarity of northern pike GH to other growth hormones

Figure 2 is a cladogram representing the infradivision *Euteleostei* (true teleosts), which is the largest of the four lines of teleost evolution and includes nearly 20,000 species. Eight fish, whose GH mRNAs have been cloned and sequenced, are indicated within their respective orders. A comparison of the amino acid sequence of these GHs, together with both piscine and overall consensus sequences, are provided in Figure 3. For comparison with land vertebrates, human, bovine, fox, and chicken GHs

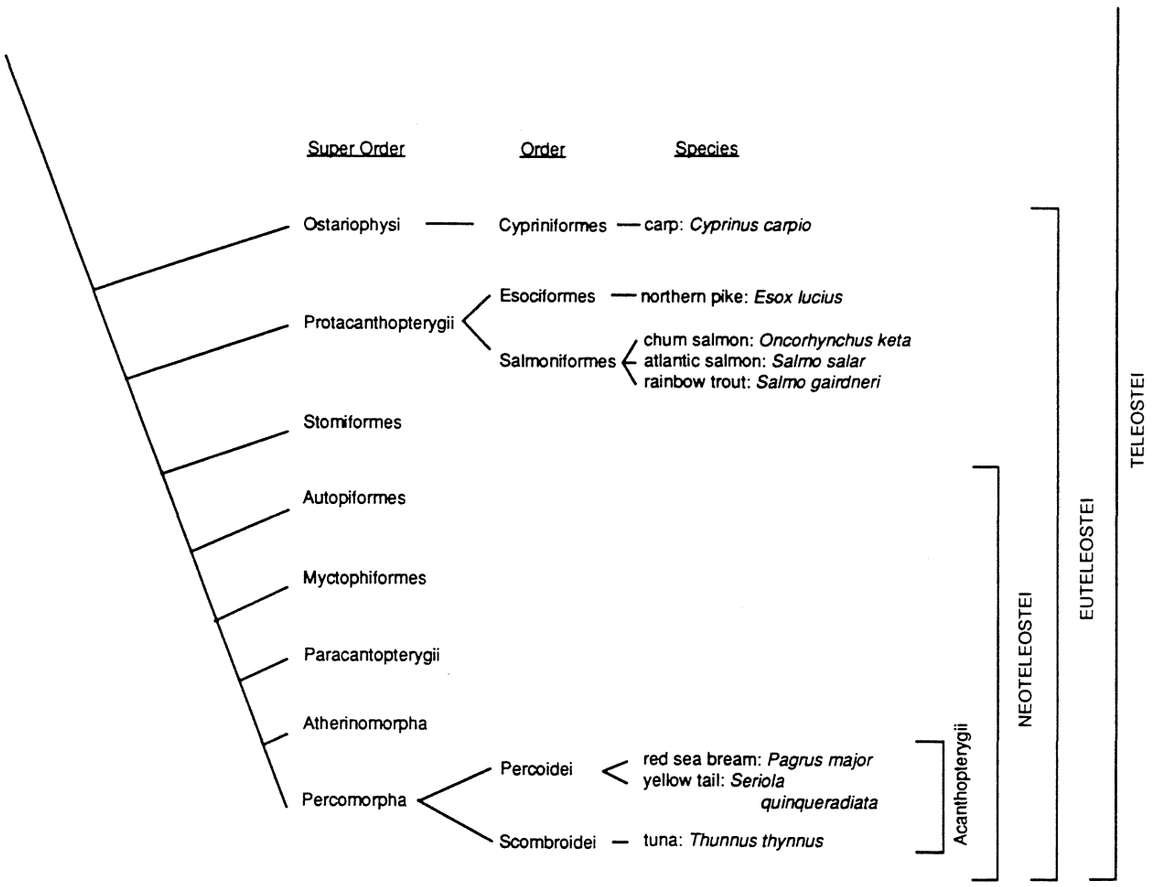


Figure 2. Cladogram depicting the phylogeny (Lauder and Liem, 1983) of the Euteleosts.

have also been included. The capital letters within the two consensus sequences indicate those residues that are conserved within all GHs respective to their consensus sequence. These conserved residues can be localized to four regions of the mature hormone: region I is amino acid residues 6–21, region II is residues 50–84, region III is residues 112–122, and region IV is residues 163–184 (numbers refer to the overall consensus). Each region is separated from the next by at least 28 aa residues. Both phenylalanine (28) and four cystiene residues (50, 166, 183, and 191) are also completely conserved. As compared to the overall consensus sequence, the conserved regions within the piscine consensus sequence are slightly expanded. Moreover, the leucines (32 and 141), asparagine (103), aspartic acid (135), and arginine (158) residues are also conserved within the piscine sequences. Conversely, residues comprising the signal peptides of each GH show the most divergence. For example, the signal peptides from carp, chicken, and bovine are completely different from the other species' GHs.

The four conserved regions probably are essential for either tertiary folding or hormone receptor interactions, or both, because they are the only areas of strong GH similarity between fish, mammals, and birds. Regions I, III, and IV are contained within different helical regions of the mature GH (helix 1, 3, and 4, respectively), whereas region II resides mostly within the loop between helix 1 and 2 (Cunningham et al., 1989). Conserved regions I, II, and IV contain all but one of the binding patch amino acid residues (residues 10, 54, 56, 58, 64, 68, 171, 172, 174, 175, 176, 178, and 182) identified as critical for binding of the human GH to its receptor (Cunningham and Wells, 1989). The only residue not included in these

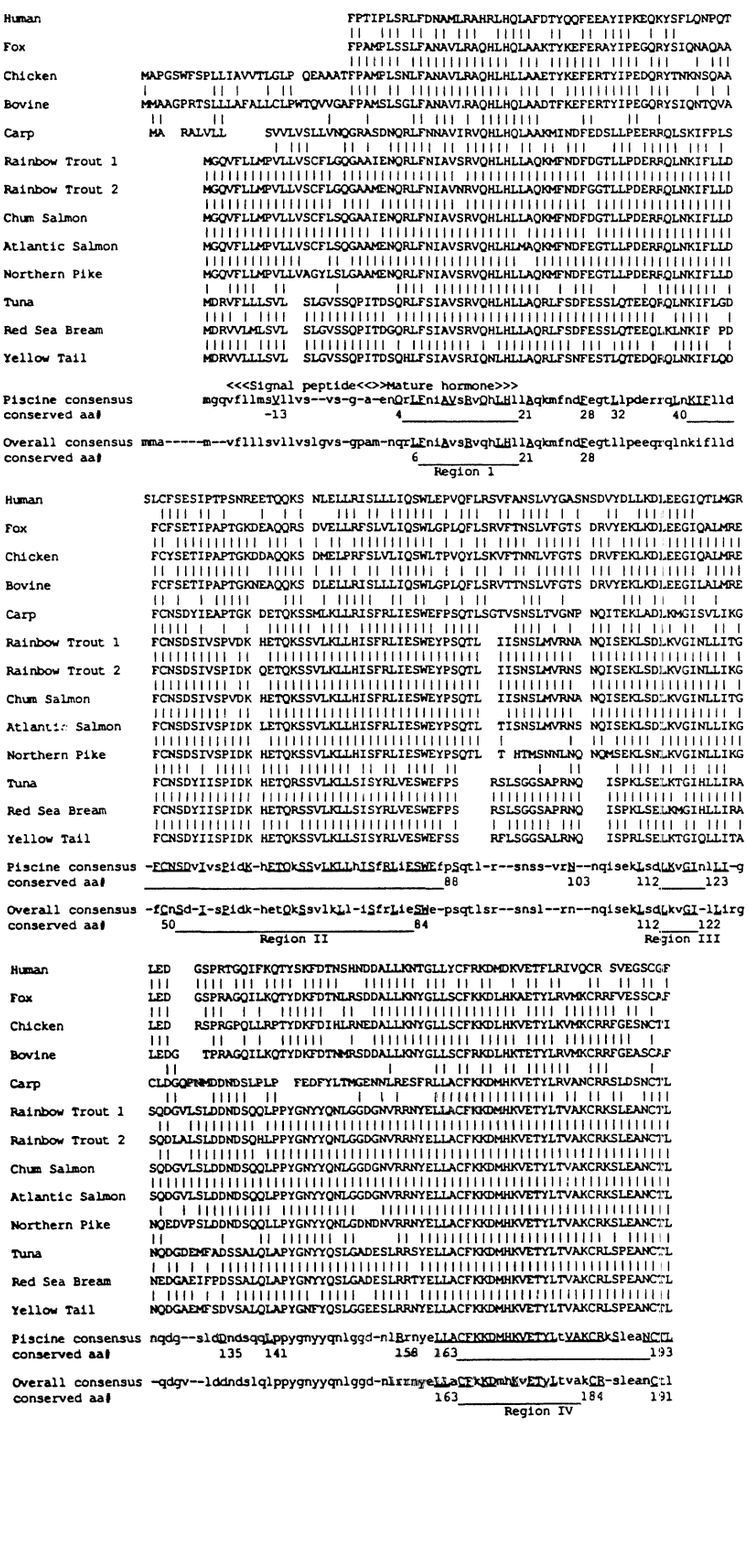


Figure 3. Pregrowth hormone amino acid sequence comparison. Two nearly identical rainbow trout growth hormone-1 genes have been isolated (Agellon and Chen, 1986; Rentier-Delrue et al., 1989). They differ at position −5 (glycine versus serine, respectively) and position 126 (serine versus asparagine, respectively). The sequence reported above is from Agellon and Chen (1986). Rainbow trout-2 is taken from Rentier-Delrue et al. (1989). Gaps in similarity are indicated by the absence of slash marks. Within the consensus sequence, capital letters (underlined) indicate 100% conservation, whereas lowercase letters correspond to mismatched residues.

regions is residue 185, which lies just outside of region IV. This residue is completely conserved within each fish superorder. Perhaps residue 185 is important for custom-fitting each GH to its own receptor.

Previously, we have shown that northern pike transgenic for bovine GH have an increased growth potential (Gross et al., 1992). Thus, although the overall GH sequences vary between the two species, bovine GH appears to function within northern pike, perhaps due to the high degree of conservation found in the regions important for hormone/receptor interaction. Consequently, further characterization of the highly conserved regions will be important to define the biological activity of GH.

The information used in developing evolutionary relationships among fish comes largely from taxonomic studies, which in large part rely on meristatic measurements. Sequencing data, as they become available, will become another data source used to refine the evolutionary relationships between individual fish species. The similarity between the fish GH amino acid sequences noted in Figure 3 are summarized in Table 1, in which the fish GHs are grouped by superorder and their amino acid sequence similarity is listed.

This sequence data correlate nicely with the original taxonomic hierarchy (see Figure 2). For example, the northern pike GH sequence is most similar to the GHs of other members of its superorder (salmon and trout, 89%), whereas the carp GH sequence is more closely associated to northern pike (66%) than either to tuna (57%), red sea bream (53%), or yellow tail (49%). The *Percomorphae* represent a large, immensely variable group of fish of over 11,000 species, accounting for approximately half of all known fish species. Although the three fish listed within this superorder are quite diverse, their amino acid similarity is still approximately 89%. Therefore, piscine GH sequence data will be useful in quantifying evolutionary divergence.

Our cloning of the northern pike GH should further enable us to investigate the structure and function of fish GHs, as well as to further our studies of growth-enhanced transgenic fish.

Experimental Procedures

Preparation of cDNA library

Total RNA from northern pike pituitary glands (both anterior and posterior) was prepared by the guanidium/cesium chloride method (Davis et al., 1986). Using this RNA as a template, we prepared a cDNA library utilizing the plasmid pCDM8(Invitrogen) and *Eschericia coli* MC1061/P3 as host strain. To detect colonies, the *Eco*R1/*Sal*1 fragment from pAF51dS, which contains the 5′ end of the rainbow trout GH cDNA (1–216 bp: kindly provided by Dr Tom Chen), was end-labeled with (^{32}P) by polynucleotide kinase and used as a probe to detect colonies on Zetabind filters under moderate stringency (40% formamide [v/v], 1.0 mol NaCl, 20 mM sodium phosphate (pH, 6.8), 0.1% SDS, 1% Denhardts, and 500 μg sheared calf thymus DNA).

DNA sequence analysis

All determinations of nucleotide sequence were performed on double-stranded plasmids. Both

Table 1. Homology among piscine mature growth hormones.[a]

	Protacanthoptery GII				Ostariophysi	Percomorpha		
	Northern pike (NP)	Atlantic salmon (AS)	Chum salmon (CS)	Rainbow trout (RT)	Carp (C)	Red sea bream (RSB)	Tuna (T)	Yellowtail (YT)
NP	100	91	89	89	66	70	67	65
AS		100	95	95	67	66	66	64
CHS			100	100	66	66	68	65
RT				100	65	65	68	66
C					100	53	57	49
RSB						100	94	85
T							100	89
YT								100

[a]Numbers show the % identity of amino acid residues between various piscine growth hormones.

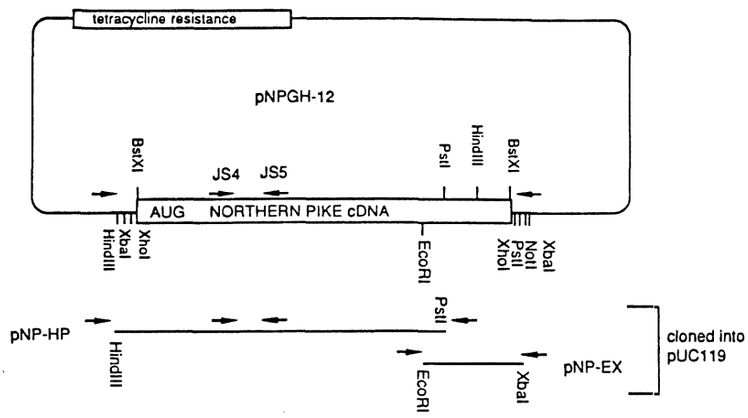


Figure 4. Restriction map of the northern pike GH cDNA insert in pNPGH-12 and its associated sequencing clones. Only the multiple cloning site and insert are shown for the pUC clones. Location of the sequencing oligonucleotide primers are indicated by *arrows*.

strands were sequenced to ensure accuracy. Two subgenomic fragments, *Hind*III to *Pst*I, containing GH sequences 1 to 1,002 bp, and *Eco*RI to *Xba*I, containing GH sequences 925 to 1,227 bp, were subcloned into pUC119 (Figure 4). The overlapping subclones were sequenced using commercial forward and reverse oligonucleotide primers complementary to the flanking vector sequences (Promega, Madison, WI). The complete cDNA, pNPGH12, was sequenced with specific oligonucleotide primers corresponding to flanking vector DNA as well as to GH sequences determined from preliminary results (JS4:GAGTCCTGGGAGTAC;JS5:GGACCACGTTTCGCAGG). Double-stranded plasmid templates were prepared for sequence analysis as described previously (Kraft et al., 1988). Approximately 7 μg of plasmid DNA was denatured in 0.18 N sodium hydroxide and 0.18 mM EDTA for 5 minutes at room temperature. The solution was neutralized with the addition of 0.27 mol NH_4Ac (pH, 4.5). The DNA was precipitated with 2 volumes ethanol, followed by an 80% ethanol wash, and dried. Sequencing reactions using (^{35}S)-labeled dATP (New England Nuclear, Boston, MA) were performed with a commercial kit (Sequenase; U.S. Biochemicals, Cleveland, OH). The sequences were determined following polyacrylamide (6% w/v) gel electrophoresis, fixation in 5% (v/v) methanol/acetic acid (1/1) in water, dehydration of the gel, and autoradiography.

Acknowledgments

This article is dedicated to the memory of Dr Kevin Guise. We would like to thank Mark Gross and Drs Eric Hallerman and Anne Kapuscinski for their help in procurement of northern pike pituitary glands. Special thanks to Dr T. T. Chen for the gift of pAF51dS. This research was supported in part by the Minnesota Sea Grant Program, NOAA Office of Sea Grant, Department of Commerce Grant No. NOAA-86AA-D-SG112 (JR283); the Legislative Commission on Minnesota Resources; the Blandin Foundation; and the Greater Minnesota Corporation.

References

Agellon, L.B., and Chen, T.T. (1986). Rainbow trout growth hormone: molecular cloning of cDNA and expression in *Escherichia coli*. *DNA* 5:463–471.

Chao, S.C., Pan, F.M., and Chang, W.C. (1989). Purification of carp growth hormone and cloning of complementary DNA. *Biochem Biophys Acta* 1007:233–236.

Cunningham, B.C., Jhurani, P., Ng, P., and Wells, J.A. (1989). Receptor and antibody epitopes in human growth hormone identified by homolog-scanning mutagenesis. *Science* 243: 1330–1334.

Cunningham, B.C., and Wells, J.A. (1989). High resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* 244:1081–1085.

Davis, L.G., Dibner, M.D., and Battey, J.E. (1986). *Basic Methods in Molecular Biology*. N.Y.: Elsevier, pp. 136–138.

Gross, M., Schneider, J.F., Moav, N., et al. (1992). Molecular analysis and growth evaluation of northern pike (*esox lucius*) microinjected with growth hormone genes. *Aquaculture* (in press).

Hew, C.L., Trinh, K.Y., Du, S.D., and Song, S. (1989). Molecular cloning and expression of salmon pituitary hormones. *Fish Physiol Biochem* 7:375–380.

Lauder, A. and Liem, K.F. (1983). *An Introduction to Ichthyology*. Cambridge, MA: Museum of Comparative Zoology, Harvard University.

Lorens, J.B., Nerland, A.H., Lossius, I., et al. (1989). Nucleotide sequence for the atlantic salmon growth hormone gene. *Nucleic Acids Res* 17:2352.

Kraft, R., Tardiff, J., Krauter, K., and Leinwand, L. (1988). Using mini-prep plasmid DNA for sequencing double standard templates with sequenase. *Biotechniques* 6:544–546.

Miller, W.L., and Eberhardt, N.L. (1983). Structure and evolution of the growth hormone gene family. *Endocr Rev* 4:97–129.

Momota, H., Kosugi, R., Hiramatsu, H., et al. (1988). Nucleotide sequence of cDNA encoding the pregrowth hormone of red sea bream (*Pagrus major*). *Nucleic Acids Res* 16:3107.

Mori, H., Kimura, T., Tsunenari, T., et al. (1989). The complete cDNA sequence for the premature form of growth hormone of the flounder (*Paralichthymus olivaceus*). *Nucleic Acids Res* 17:3977.

Niall, H.D., Hogan, M.I., Sayer, R., et al. (1971). Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene duplication. *Proc Natl Acad Sci USA* 68:866–870.

Nicoll, C.S., Steiny, S.S., King, D.S., et al. (1987). The primary structure of coho salmon growth hormone and its cDNA. *Gen Comp Endocrinol* 68:387–399.

Rentier-Delrue, F., Swennen, D., Mercier, L., et al. (1989). Molecular cloning and characterization of two forms of trout growth hormone cDNA: expression and secretion of tGH-II by *Escherichia coli*. *DNA* 8:109–116.

Sato, N., Watanabe, K., Murato, K., et al. (1988). Molecular cloning and nucleotide sequence of tuna growth hormone cDNA. *Biochim Biophys Acta* 949:35–42.

Sekine, S., Mizukami, T., Nishi, T., et al. (1985). Cloning and expression of cDNA for salmon growth hormones in *Escherchia coli*. *Proc Natl Acad Sci USA* 82:4306–4310.

Watahika, M., Tanaka, M., Masuda, N., et al. (1988). cDNA cloning and primary structure of yellowtail *Seriola quinqueradiata* pregrowth hormone. *Gen Comp Endocrinol* 70:401–406.

Molecular Marine Biology and Biotechnology (1992) 1(2), 125–135

Characterization of *Alu*I repeats of zebrafish (*Brachydanio rerio*)*

Ling He
Department of Fisheries and Wildlife, University of Minnesota, St Paul, Minnesota 55108, U.S.A.

Zuoyan Zhu
Institute of Hydrobiology, Academia Sinica, Wuhan, Hubei, P.R. China

Anthony J. Faras*
Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, U.S.A.

Kevin S. Guise†
Department of Animal Science, University of Minnesota, St Paul, Minnesota 55108, U.S.A.

Perry B. Hackett*‡
Department of Genetics and Cell Biology, University of Minnesota, St Paul, and Institute of Human Genetics, University of Minnesota, Minneapolis, Minnesota 55455, U.S.A.

Anne R. Kapuscinski§
Department of Fisheries and Wildlife, University of Minnesota, St Paul, Minnesota 55108, U.S.A.

Abstract

Two families of repetitive DNA sequences were isolated from the zebrafish genome and characterized. Eight different sequences were sequenced and classified by two standards, their (G + C) composition and their lengths. For convenience, the sequences were first divided into two types. Type I was (A + T)-rich, was repeated approximately 500,000 times, and constituted approximately 5% of the zebrafish genome. Type II was (G + C)-rich, was reiterated approximately 90,000 times, and comprised approximately 0.5% of the genome. Agarose gel electrophoresis of zebrafish DNA cleaved with *Alu*I revealed three distinguishable bands of repetitive fragments: large (approximately 180 bp, designated RFAL), medium (approximately 140 bp, RFAM), and small (approximately 90 bp, RFAS). The RFAL fragments contained both type I and type II sequences. Limited digestion of genomic DNA indicated that RFAL and RFAM were tandemly arranged in the genome, whereas RFAS showed a mixed pattern of both tandem and interspersed repeated arrangements. Although inclusion of a repetitive sequence in a transgenic construct did not appreciably accelerate homologous integration of transgenes into the zebrafish genome, the *Alu*I sequences could facilitate transgene mapping following chromosomal integration.

Introduction

The insertion of genes into piscine genomes is being conducted in an increasing number of laboratories worldwide as appreciation increases for the value of transgenic fish for studies of the molecular biology of vertebrate development (Powers, 1989) and aquaculture (Maclean and Penman, 1990). Microinjection of exogenous DNA into the nuclei of fertilized eggs has been the only successful technique for creating transgenic fish (Hew, 1989). A major problem is that the genes are usually microinjected into the cytoplasm of fertilized eggs rather than into their pronuclei. During the course of embryogenesis, the injected DNA may integrate randomly into genomes of different cells at different chromosomal locations (Zhu et al., 1989; Stuart et al., 1988; Hallerman et al., 1990). Consequently, most transgenic fish are mosaic.

To overcome the problem of mosaicism and to enhance the integration rate of foreign genes into fish genomes, we examined the possibility of using repetitive DNA sequences to facilitate homologous recombination. In higher organisms, highly and moderately repeated sequences are reiterated 10^3 to 10^6 times in the genome. These sequences account for 10 to 70% of the total genomes of different

* This author is also affiliated with the Institute of Human Genetics, University of Minnesota.

†Deceased February, 1991.

‡ Correspondence should be sent to this author.

§ Article 19724 of the Minnesota Agricultural Experiment Station Scientific Journal Article Series.

species (Corneo et al., 1967; Birnstiel et al., 1968; Hatch and Mazrimas, 1970; Davidson and Britten, 1973; Fowler and Skinner, 1985). Repetitive DNA sequences are likely to have an important role in chromosomal organization (John and Miklos, 1979; Jelinek and Schmid, 1982). The *Alu* family is the most common repeated DNA sequence in the human genome, consisting of approximately a million copies of approximately 300 base pairs (bp) (Houck et al., 1979), and similar sequences have been found in many species (Schmidt and Jelinek, 1982).

Consequently, we initiated a study of *Alu*-repetitive elements in zebrafish genomes for their eventual application in the transfer of genes into fish. Zebrafish (*Brachydanio rerio*) is an excellent model species (Streisinger et al., 1981; Kimmel and Law, 1985a, b, c) to rapidly evaluate basic problems in gene transfer studies (Stuart et al., 1988; Liu et al., 1990). We cloned and characterized several *Alu*I repeats from zebrafish. We also examined their usefulness in facilitating integration of transgenes into the genomes of fertilized zebrafish eggs.

Results and Discussion

Repetitive DNA sequences in the zebrafish genome

Zebrafish genomic DNA was digested with 22 different restriction endonucleases to detect the existence of repetitive sequences; patterns produced by 12 of these enzymes are shown in Figure 1. These digestions generated four repetitive fragments (RF) designated as RFAL (*Alu*I large band, approximately 180 bp), RFAM (*Alu*I medium band, approximately 140 bp), RFAS (*Alu*I small band, approximately 90 bp); and RFM (*Mbo*I *Sau*3A band, approximately 200 bp). A few fine bands, varying in size from 200 bp to 1,000 bp, were also revealed on lanes containing *Hin*dIII- and *Bam*HI-digested DNA.

We initially focused our cloning on the three detected *Alu*I restriction bands. The blunt-ended *Alu* fragments were cloned into the plasmid pUC118 vector at the *Sma*I site, and 36 recombinant clones were randomly picked from ligation reactions containing each of the three *Alu*I bands. Eight clones of the 108 clones containing *Alu*I repetitive fragments were selected for further analysis. Six clones were derived from the large band (RFAL-1, RFAL-2, RFAL-3, RFAL-4, RFAL-5, and RFAL-6), and the RFAM and RFAS clones came from the medium and small bands, respectively.

Nucleotide sequence analysis

Nucleotide sequences of the eight selected repetitive fragments are shown in Figure 2. The six fragments cloned from the large band of *Alu*I digestion (RFALs 1–6) were similar to each other in size (181–185 bp) but different in their nucleotide sequences. RFAM and RFAS were 141 bp and 92 bp in length, respectively. Comparisons of (A + T) and (G + C) compositions among the eight *Alu*I repetitive fragments (Table 1) showed that RFAL-1, RFAL-2, RFAL-3, and RFAM were more than 61% (A + T), in contrast to RFAL-4, RFAL-5, RFAL-6, and RFAS, which were less than 36% (A + T). Fragments within each type of repetitive sequence had homologous, but not identical, nucleotide sequences (Table 2, Figure 3). These results on (A + T) composition and sequence

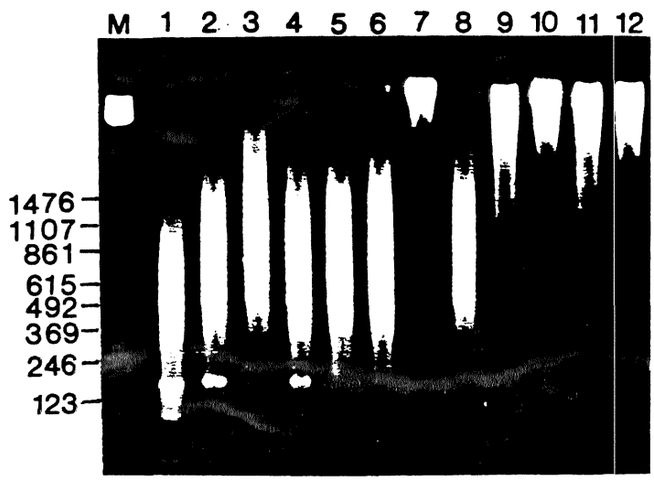


Figure 1. Restriction endonuclease digestion profiles of zebrafish DNA following electrophoresis through a 1.5% agarose gel: (1) *Alu*I, (2) *Mbo*I, (3) *Hae*III, (4) *Sau*3A, (5) *Hin*fI, (6) *Rsa*I, (7) *Sal*I, (8) *Mbo*II, (9) *Hin*dIII, (10) *Bam*HI, (11) *Bgl*II, (12) *Eco*RI, (M) marker consisting of 123-bp ladder.

Figure 2. DNA sequences of eight clones of zebrafish repetitive elements. *Underlining* indicates internal repeats; *asterisks* indicate mismatched base pairs; a-a′, b-b′, and c-c′ represent direct repeats in each repetitive fragment. In RFAL-6, *letters in bold* represent a pair of inverted repeats. These DNA sequences have been deposited in GenBank under accession numbers M83121–83128.

```
RFAL-1    CTCATTTTCA ACGTTCAATT TAGAATGTGA TAAAACCAGT TCCAGCCACT AAAAA
                     * a
GCATTATCTT TTTGTTTTAG ACAACATTTC ATGCACTGTT AAACATGTTA AAGCAAGTTG CAAGT
                                                        a' *
GAAAATCTAT GTCTCTGACT GAGTTTGCAT TACTGCTTAT TTTGACCTCT GCTGGCTCTG AAAAG
           b *                                      b'
RFAL-2    CTCATTTTCA ACGTCCAATT CAGAATGTAA TAAAACCAGA TCCAGCCATA AAATG
                     * a
CATCATTCTT TTTTGTTTTA GACAACATTT CATGCACTGT TAAACATGTT AAAGCAAGTT GCAAG
                                                        a' * *
TGAAAATCTA TGTCTCTGAC TGAGTGCATT ACTGTGATTT GACCTCTCTG CTGGCTGAGA TAAG
           b *                                      b'
RFAL-3    CTCATTTTCA ACGTCCAATT CAGAATGATC CAGCCATAAA ATGCATCATT CTTTT
                     * a
TTGTTTTAGA CAACAACAAT TCATGCACTG TTAAACATGT TAAAGCAAGT TGCAAGTGAA AATCT
                                                   a'  **
ATGTCTCTGA CTGAGTTTGC ACCACTGTGA TTTGACCTCT CTGCTGCTGG CTGGCTGAGA TAAG
      b *                                    b'

RFAM      CTATAAAATG CATCATTCTT TTTTGTTTTA GACAACATTT CATGCACTGT TAAAC

ATGTTAAAGC AAGTTGCAAG TGAAAATCTA TGTCTCTGAC TGAGTTTGCA TTACTGTGAT TTGAC
                                        a  *
CTCTCTGCTG GCTGAGATAA G
     a'

RFAL-4    CTACCCAGGG TCCACCTCTC TCCCTGGACT TGCAGTCTAG GCCCAAGGGT GCCCC
                                   a              **      * *
TGGCATCCAC CTGGGGGCCT CTGACCCGAC AATTTACCCA GGGGTCCACC TCTCTCCCTG GACTT
             * b *                                 *                  a'
GCAGTGGAGG CCTATGGGTG CCCCCTTGCC CTCCACCTAG GGGTCTCTGC CACTGAGCAA G
    **     * *                              * b' *
RFAL-5    CTACCCAGGG TCCACCTCTC TACATGGACT TGCAGTAGAG GCCCCAGGGT GCCCC
                    a                                 b
CTGGCCCTCC ACCTAGGGGC CTCTGACCCC AGCAAGATAC CCAGAGGTCC ACCTCTCTCC CTGGA
      **     c              *                *   a'
CCTTGCAGTA GAGGCCCAGA GTGCCCCCTG GCCCATCACC TAGGGGCCTC TGACCCAGCA AG
     b'                          **     c'
RFAL-6    CTACCCTGGG GTCCACGTCT CTTCCCGGAC TTGCAGTAGA GGCCCCAGGG TGCCC
                 * a                          b       *
CCTGGCCCTC CACCTAGGGG CCTAAGACCC CAACAAGATA CCCAGGGGTC CACCTCTCTC CCTGG
                   c    **      *          *  a'                  *
ACTTGCAGTA GAGGCCCTAGG AAACCTCTT GGCCCTCCAC CTAGGGGCCT CTGACCCCAG
     b'          *                                * c'     **       *
CAAG

RFAS      CTACCCAGGG GTCCACAACT CTCCCTGGAC TTGCAGTAGA GGCCCCAGGG TGCCC

CCTGGCCCTC CACCTAGTGG CCTAAGACCC CAGCAAG
```

Table 1. Summary of the contents of the repetitive sequences.

Clone	Length	(A + T) (%)
RFAL-1	185 bp	121 bp (65%)
RFAL-2	184 bp	118 bp (64%)
RFAL-3	184 bp	113 bp (61%)
RFAM	141 bp	92 bp (65%)
RFAL-4	181 bp	65 bp (36%)
RFAL-5	182 bp	64 bp (35%)
RFAL-6	184 bp	66 bp (36%)
RFAS	92 bp	32 bp (35%)

Table 2. Percent identity between cloned repetitive sequences.

Clone	AL-2	AL-3	AM	AL-4	AL-5	AL-6	AS
RFAL-1	89.6	71.8	87.8	35.6	36.3	37.5	40.2
RFAL-2	---	98.3	99.3	35.8	30.8	33.5	38.1
RFAL-3		---	97.2	40.1	35.1	39.9	41.3
RFAM			---	37.6	34.8	30.5	41.3
RFAL-4				---	77.8	81.5	84.5
RFAL-5					---	85.7	86.8
RFAL-6						---	92.4
RFAS							---

Type I:

```
                                                                    bp
RFAL-1    CTCATTTTCAACGTTCAATTTAGAATGTGATAAAACCAGTTCCAGCCACT        50
RFAL-2    ..............c.....c.......a..........a.........*.       49
RFAL-3    ..............c.....c......***********a.........*.       37
RFAM                                                    .ta.       4

AAAAAGCATTAT*CTTTTT*GTTTTAGACAACATTTCATGCA***CTGTTAAACATGTTAAAG   108
....t....c..t......t.....................***.................   109
....t....c..t......t.............acaat.catgca.................   100
....t....c..t......t.....................***.................    64

CAAGTTGCAAGTGAAAATCTATGTCTCTGACTGAGTTTGCATTACTGCTTATTTTGACCTCT*   170
.....................................**.........*.g....*.......c   168
.........................................cc....tg*....*.......c   161
.............................................*.g.*..........c   125

*GCTGGCTCT********GAAAAG                                         185
t.......ga********..t...                                         184
t....*..ggctggctga..t...                                         184
t.......ga********..t...                                         141
```

Type II:

```
                                                                    bp
RFAL-4    CTACCCAGGG*TCCACCTCTCT*CCCTGGACTTGCAGTCTAGGCCCAAGG        48
RFAL-5    ..........*...........*a.a............ag......c...       48
RFAL-6    ......t...g.....g.....t...*...........ag......c...       49
RFAS      ..........g.....aa....*...............ag......c...       49

GTGCCCC*TGGC*ATCCACCTGGGGGCCTCTGACCCG*ACAATT*TACCCAGGGGTCCACCTC   107
.......c....cc........a..............c*.gc.aga.......a.........   110
.......c....cc........a.......aa.....ca....*ga.................   111
.....................a.t.............*.gc.ag                      92

TCTCCCTGGAC*TTGCAGTGGAGGCC*TATGGGTGCCCCCTTG*CCCTCCACCTAGGGGTCTC   167
..........c.......a......*c.ga*.........g.*...at.........c...   170
..........*.......a......c..*..aaa..*t....g..............c...   171


TGCCACTGAGCAAG                                                   181
..a.c.**......                                                   182
..a.c.c*......                                                   184
```

Figure 3. Comparisons of the *AluI* repetitive sequences. *Asterisks* indicate deletion of nucleotides; *dots between letters* indicate conserved nucleotides.

homology suggest that there are at least two types of repetitive fragments. Such heterogeneity of highly repeated sequences is the basis of the ubiquitous intermediate reassociation kinetics of fragmented vertebrate DNA (Darnell et al., 1986). Most short interspersed sequence (SINES) families in vertebrate chromatin diverge from a consensus sequence by 8 to 30% (Weiner et al., 1986), which is approximately what we found for the two families of *AluI* sequences in zebrafish.

Fragments of the same type had characteristic internal repeats but with different lengths and patterns within every fragment (see Figure 2). For example, an attribute common to three members of type II, RFAL-4, RFAL-5, and RFAL-6, was a large (>35 bp), direct internal repeat with a few mismatched nucleotides. Each member also had unique attributes. RFAL-4 had two direct internal repeats of 55 bp and 18 bp, including 7 mismatched base pairs. RFAL-5 had three different lengths of direct internal repeats, 43 bp, 20 bp, and 16 bp, with four mismatched base pairs. The cumulative length of the direct internal repeats in RFAL-5 was 158 bp, 87% of its total length. Similarly, three internal repeats were found in the RFAL-6 repetitive fragment. However, there was a pair of 12 bp inverted repeats that were not found in the other repeated elements. The fourth member of the type II elements, RFAS, did not have any direct repeats; however, if RFAL-6 is divided into two equal fragments from the central point, the similarities of these two fragments with RFAS are 92 and 87%. Thus, RFAS resembled a unit sequence that was apparently doubled in the other type II elements. Additional scrutiny revealed a 20-bp sequence (TACCCAGGGTCCACCTCTCT) repeated seven times in the four members of the type II repetitive sequences. No long internal repeats were found in type I repetitive sequences, but there were some short direct repeats (up to 10 bp) in each member (see Figure 2).

Genomic copy numbers for each type of repetitive sequence

Members within each type of *AluI* repetitive sequence shared 72% or more of their nucleotide sequences (Table 2) and were sufficiently similar to cross-hybridize with one another under stringent conditions. Consequently, it was impossible to calculate precisely the copy numbers of each repeat in the genome simply by DNA hybridization. However, when any one of the eight repetitive fragments served as a radioactive probe, copy numbers deduced by dot-blot hybridizations could be estimated for this type of repetitive sequence. Accordingly, RFAM and RFAS were used to estimate the copy numbers of type I and type II repetitive sequences in the zebrafish genome (Figure 4). This estimation was done by determining the extent of hybridization of a probe for repetitive sequences type I or II hybridized to itself (see lanes designated B, Figure 4) and to genomic DNA (see lanes designated A, Figure 4). Hybridization was quantified by cutting out the dots, counting the radioactivity in a liquid scintillation counter, and performing linear regression analysis on the data for each dilution series to determine accurately the relative hybridizations of the probes with their homologues.

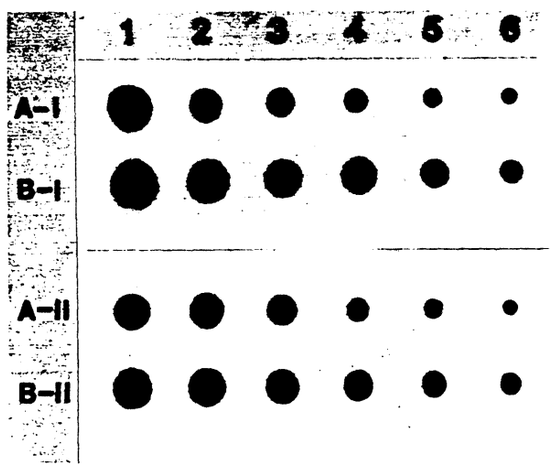


Figure 4. Quantitative analysis of repetitive sequences in the zebrafish genome. DNA dot blots for rows A-I and A-II contained genomic DNA diluted stepwise by 50% from 1,920 to 960, 480, 240, 120, and 60 ng in lanes 1–6. Rows B-I and B-II show DNA samples of cloned type I and type II sequences, respectively; lanes 1–6 represent DNA diluted stepwise by 50% from 32 to 16, 8, 4, 2, and 1 ng. A [^{32}P]-labeled RFAM probe was hybridized with DNA samples in rows A-I and B-I, and labeled RFAS probe was hybridized with DNA samples in rows A-II and B-II. Probes contained the repeated fragment plus 21 bp of pUC118 vector.

The results indicated that for the type I (G/C-rich) repeated elements, the RFAM probe had equivalent hybridization to 1 ng of pure probe and 25 ng of genomic DNA, indicating that approximately 4% of the genome is type I repetitive sequence. From Figure 1 it is evident that the RFAL sequences are approximately two-fold more prevalent than the RFAM sequences. Accordingly, a correction factor (1.2) that took into account the relative lengths and percentage contribution of the RFAL and RFAM fragments was calculated. Multiplying the 1.2 correction factor by the initial value of 4% indicates that type I repetitive sequences are approximately 5% of the zebrafish genome. Likewise, the A/T-rich type II sequences comprise only approximately 0.5% of the zebrafish genome. In obtaining the values for the type II sequences, the length correction is not necessary because the type II sequences are essentially dimers of the RFAS sequence. The zebrafish genome is approximately 1.6×10^9 bp/haploid genome (Hinegardner, 1968). Consequently, type I sequences comprising 5% of the genome represent approximately 8×10^7 bp or approximately 5×10^5 sequences that are 170 bp long. Similarly, the 90-bp, unit length type II sequences that make up 0.5% of the genome (8×10^6 bp) would be reiterated approximately 9×10^4-fold in a haploid zebrafish genome, as often dimeric sequences.

Repeated patterns of AluI *fragments in the genome*

If copy numbers of a repetitive fragment in the genome exceed 1 million in a mammalian genome of 3×10^9 bp, the fragment is generally categorized as a highly repetitive sequence. Such sequences are usually tandemly concentrated in centromeric and telomeric heterochromatin and are not transcribed (Long and Dawid, 1980). By this criterion, zebrafish type I repeated fragments could be categorized as highly repetitive sequences if they existed in tandemly repeated sequences.

The possibility of tandemly arranged repeated elements was examined by digestion of genomic DNA with limiting concentrations of deoxyribonuclease (DNase) (Figure 5). Patterns of Southern blot hybridizations against radioactive probes of RFAL, RFAM, and RFAS showed that as the concentration of *AluI* decreased (right to left), the size of the genomic DNA bands increased in a stepwise fashion (see Figure 5). Both RFAL and RFAM (type I) probes revealed almost the same pattern of hybridization,

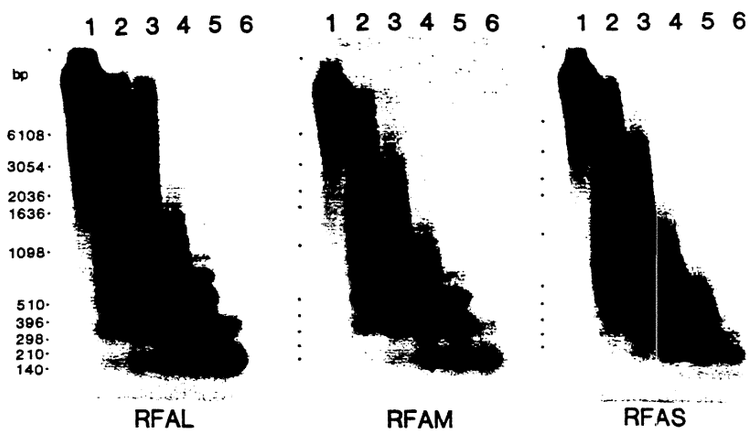


Figure 5. Tandem orientation of repetitive sequences in the zebrafish genome. Zebrafish genomic DNA was cleaved with increasing amounts of *Alu*I, isolated, separated on a 1.0% agarose gel, and analyzed by Southern hybridization. Lanes 1–6 of the autoradiogram were loaded with 6.1 μg of zebrafish genomic DNA and digested at 37°C for 1 hour with increasing amounts of restriction endonuclease *Alu*I as follows: (1) 0 U, (2) 0.0015 U, (3) 0.003 U, (4) 0.006 U, (5) 0.012 U, and (6) 6 U, respectively. Three Southern blots, made in the same way, were hybridized with [^{32}P]-labeled RFAL, RFAM, or RFAS probes.

indicating that many of the repetitive elements were tandemly arranged in the genome. However, the RFAS (type II) probe revealed a more mixed pattern, including tandem and interspersed repeated arrangements in the genome. Zebrafish type II repeated fragments are typical of SINES that exist in most animal genomes (Schmid and Jelinek, 1982; Singer, 1982). Hybridization patterns with the RFAS probe showed a smeared hybridization pattern, suggesting the presence of dispersed elements mixed with tandemly arranged repetitive elements. Figure 6 shows the apparent relationship of the eight clones we characterized. Previous studies (Stumph et al., 1983) indicate that type I repeated sequences may have some role in chromosomal organization. Interspersed repetitive sequences, like our type II, may be useful for mapping genes as described herein.

Possible application in evolutionary studies

An *Alu* family of repetitive sequences exists in mammals (Krayev et al., 1982; Page et al., 1981; Weiner et al., 1986). Computer alignment studies do not suggest homology between the *Alu* family in mammals and types I and II *Alu*I repeats of zebrafish, indicating that comparisons of homology of repetitive sequences are not useful for determining evolutionary relationships of different species. Previously, we investigated the genomic DNAs of more than 20 species of the family Cyprinidae by dot-blot hybridization with a middle-repetitive sequence probe from genomic DNA of mirror carp (*Cyprinus carpio*) digested with *Hin*dIII (He et al., 1987). For *Acanthobrama simoni, Aristichthys nobilis, Carassius auratus auratus, Coreius guichenoti, Ctenopharyngodon idellus, Ochetobius elongatus, Hypophthalmichthys molitrix, Mylopharyngodon piceus, Rhinogobio cylindricus, Rhinogobio ventralis,* and *Sinilabeo decorus tungting,* we found no evidence of sequence homology. However, within three varieties of *Cyprinus carpio* (common carp, red carp, and mirror carp) the *Hin*dIII repetitive sequences were similar in sequence and in copy number. In contrast, Borchsenius and Chernov (1988) found that *Sau*3A repetitive DNA sequences of the sockeye salmon

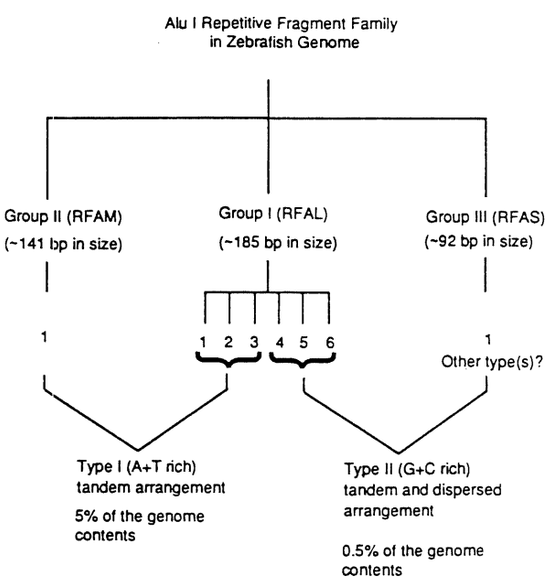


Figure 6. Proposed relationship of repetitive sequences in the zebrafish genome, based on characterization of eight clones.

(*Oncorhynchus nerka*) genome were different in anadromous and dwarf forms, suggesting that variability in the number of repetitive DNA sequences was useful for discrimination of these two different forms (Kirpichnikov et al., 1990). These results indicate that analysis of repeated sequences may provide useful information about evolutionary relationships in closely related fish. The repeated sequence "clock" may be a useful adjunct to the commonly used mitochondrial clock. The evolution of SINE sequences in vertebrate genomes is apparently due to nonviral retroposon activity (Weiner et al., 1986). Analyses in several species suggest that a few founder sequences, presumably all related to a single progenitor sequence, have been amplified by reverse transcription of RNA followed by insertion into the genome. Gene fusion may account for the tandem arrangement of some of the sequences.

Assay of repetitive sequences as an integration element for transgenic studies

Because the rate of mosaicism is often high in transgenic fish as compared with other transgenic animals, we examined the possibility of using endogenous repetitive sequences as facilitators of foreign-gene integration into the zebrafish genome during the first few cell division cycles. We are not aware of any prior use of such a strategy. In previous studies, a construct of pRSV-CAT was employed as a foreign gene to generate transgenic goldfish (*C.auratus;* Hallerman et al., 1990). In this study, both type I and type II repetitive sequences were inserted into pRSV-CAT generating constructs pRSV-CAT(I) and pRSV-CAT(II), respectively (Figure 7). The strategy was as follows. Approximately 10^6 to 10^7 copies of DNA were microinjected per zygote. Should integration of microinjected DNA occur during the first few cell cycles, then the construct would be amplified during each round of cell division so there would be more than 10^6 copies per zygote after the gastrula stage. By analyzing the DNA in 9- to 25-hour-old embryos that developed from microinjected eggs, we could determine whether early integration occurred because the hybridization signal of a single integration event on a Southern blot would be comparable to the levels of unintegrated DNA. Accordingly,

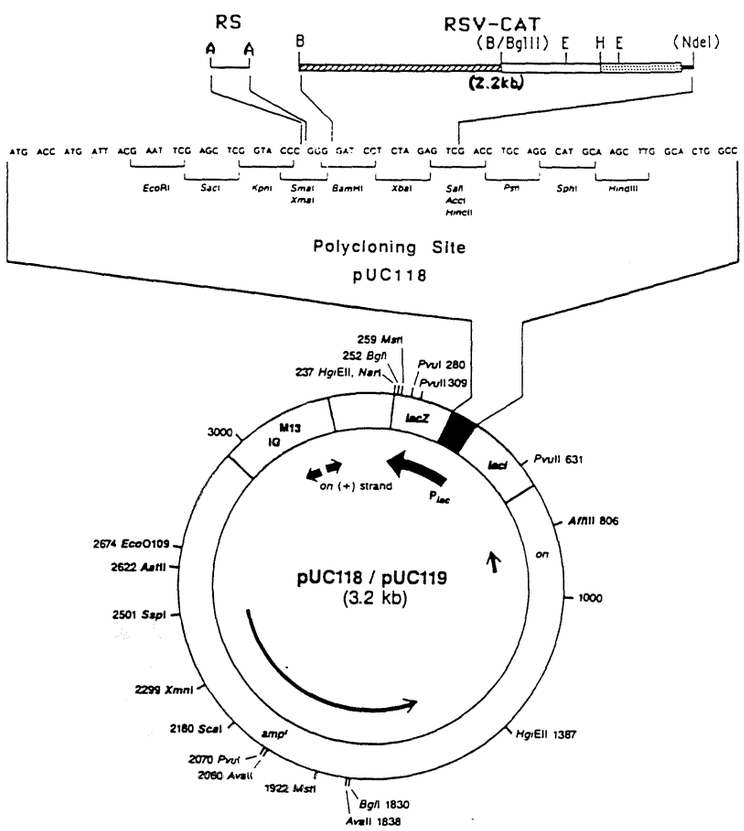


Figure 7. Expression constructs containing *AluI* repetitive sequences. This figure shows the parental construct, pRSV-CAT, which has the Rous sarcoma virus (RSV) enhancer/promoter elements from the viral long terminal repeat and the *E. coli* chloramphenicol acetyltransferase (CAT) reporter gene inserted into pUC118 (Liu et al., 1990). RSV-CAT(I) has the 141 bp RFAM repetitive sequence inserted in the *SmaI* site designated by the region flanked with As; RSV-CAT(II) has the 92 bp RFAS repetitive sequence in the region flanked with As. Symbols for restriction endonuclease sites are: A = *Alu*I; B = *Bam*HI; E = *Eco*RI; H = *Hin*dIII. Positions of *lacI*, *lacZ*, M13 intergenic region (IG, for single-stranded DNA production), plasmid origin of replication (*ori*), and amp^r gene are indicated in the parental plasmid.

either one of these two constructs or the parental plasmid pRSV-CAT was microinjected into fertilized zebrafish eggs. When embryos developed to the late gastrula (9 hr) or heart-beating (25 hr) stages, their DNA was isolated and examined by Southern blotting, using [^{32}P]-labeled pRSV-CAT as a probe.

The data in Figure 8A did not suggest unequivocally that any of the three constructs were present as integrated molecules at the late gastrula stage, although one sample from the group pRSV-CAT(I) (lane 4) and two samples from the group pRSV-CAT(II) (lanes 6 and 7) showed hybridization to high molecular weight, heterodispersed DNA. At the heart-beating stage, DNA samples from embryos injected with pRSV-CAT (lanes 10, 11, and 12) generated weak hybridization signals, whereas DNA from zygotes injected with pRSV-CAT(I) (lane 14) and pRSV-CAT(II) (lanes 15 and 16) showed stronger hybridization signals. The repeated sequences may have enhanced foreign-gene stability during zebrafish embryogenesis.

Generally, restriction endonuclease *Eco*RI diges-

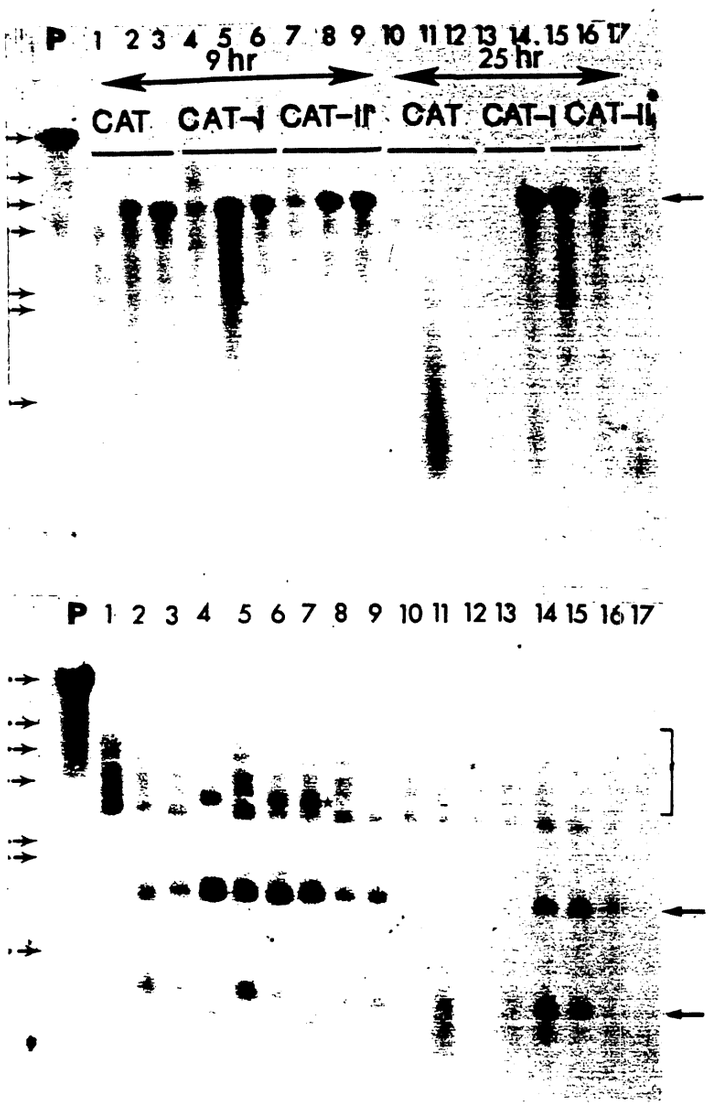


Figure 8. Analysis of transgene integration into zebrafish genomes. Fertilized zebrafish eggs were injected with either of three CAT gene expression constructs (see Figure 6). For each sample, genomic DNA was isolated from 10 embryos, 1 microinjected embryo, plus 9 noninjected control embryos to provide sufficient DNA for analysis, and divided into two portions. Five μg of the first portion was analyzed without digestion (A) and 5 μg of the second portion was cleaved with *Eco*RI and analyzed on a 1.0% agarose gel. Following electrophoresis, the samples were blotted and probed with [^{32}P]-labeled RSV-CAT construct (see Figure 6). Lanes 1–9 = DNA from 9-hour gastrula state; lanes 10–17 = DNA from 25-hour heart-beat stage of zebrafish development. Three embryos were injected for each construct. Lanes 1–3 and 10–12 = pRSV-CAT construct; lanes 4–6 and 13–14 = pRSV-CAT(I); lanes 7–9 and 15–17 = pRSV-CAT(II). The *arrows in the left margin* show the positions of λDNA cleaved with *Hin*dIII for size markers. The *arrows in the right margin* show the positions of unintegrated plasmid (intact in A; *Eco*RI-digested in B); *brackets* indicate high molecular weight DNA (A); *asterisks* indicate unusual *Eco*RI fragments, possibly due to transgene integration.

tion and Southern blotting (see Figure 8B) revealed that the three constructs existed as extrachromosomal episomes in embryos at both stages. However, the presence of bands of lighter intensity indicate that a portion of the transgenic DNA shown in lanes 4, 6, and 7 is rearranged. Foreign-gene integration is known to occur at late stages in fish embryogenesis (Stuart et al., 1988; Zhu et al., 1989). These preliminary results indicate that endogenous repetitive sequences may stabilize transgenic constructs during early embryonic development and may enhance foreign-gene integration only at a low rate in late stages of embryogenesis. Additional study of this possibility is under way.

Identification of *Alu*I repeats in the zebrafish genome may benefit integrated transgene mapping, as well as normal cellular gene mapping. Although our data indicate that many of the repeated elements are tandemly arranged, many may be singly interspersed in the zebrafish genome. Estimates for the average dispersion of *Alu*I sequences in primates is approximately 6 Kb, although the interspersion distance may vary considerably (Schmid and Jelinek, 1982; Nelson et al., 1989). Thus, using a zebrafish RFA probe in concert with either probes from the transgene of interest, or a normal cellular gene, an *Alu* polymerase chain reaction may be conducted that will amplify the chromosomal DNA flanked by the transgene and closest homologous *Alu*I site (Nelson et al., 1989). The amplified zebrafish sequence, minus the repetitive DNA portion, can be used to demonstrate integration by hybridization to normal zebrafish chromatin from control fish.

Experimental Procedures

Cloning of Alu*I repetitive DNA fragments*

Isolation of high molecular weight DNA from zebrafish zygotes was done as described by Blin and Stafford (1976) and Maniatis et al. (1982). Each sample of DNA was completely digested with *Alu*I and subjected to electrophoresis through a 6% (w/v) polyacrylamide gel (29:1 = acrylamide:N,N'-methylenebisacrylamide). Three DNA fragments were recovered using a modification of the method described by Dretzen et al. (1981). The purified DNA fragments were ligated into the *Sma*I site polylinker region of pUC118. Ligated DNA solutions were then transformed into competent cells of *Eschericia coli* strain JM101 (Hanahan, 1985).

DNA blotting

Dot blotting Zebrafish DNA samples of different concentrations were denatured by mixing with an equal volume of 0.8 N NaOH and kept at room temperature for 10 minutes. An equal volume of ice-cold 2 M NH_4Ac was added, vortexed, and the tubes were stored at 4°C for neutralization. The mixed DNA solution was slowly loaded onto a nitrocellulose filter prewashed with 100 ml of 1 M NH_4Ac. After two washings with 100 ml of 1 M NH_4Ac, the filter was dried at room temperature and baked at 80°C for 2 hours prior to hybridization.

Southern blotting Genomic DNA samples were digested either to completion with excess amounts of the restriction endonuclease *Alu*I or, for studies of the tandem arrangement of the repeated DNA sequences, with increasing concentrations of enzyme. The resulting DNA fragments were separated by size by electrophoresis through 1.5% agarose gels. The DNA in each gel was denatured, reneutralized, and transferred to a nitrocellulose membrane for analysis according to Southern (1975). The baked nitrocellulose filters were prehybridized in a plastic bag with a minimal volume of solution (5–10 ml) of 1× Denhardt's solution, 5× SSC (450 mM NaCl and 45 mM sodium citrate), 50% (v/v) formamide, 100 mg/ml yeast tRNA, and 20 mM phosphate buffer (pH, 6.5) for 5 to 6 hours at 42°C. Following prehybridization, approximately 5×10^6 cpm/ml of a [^{32}P]-labeled denatured probe was added to the prehybridization solution and hybridized at 42°C overnight. The filters were washed twice in 2× SSC and 0.5% (w/v) sodium dodecyl sulfate (SDS) for 15 minutes at room temperature and twice in a solution of 0.1× SSC and 0.5% SDS at 68°C for 30 minutes each. Wet filters were placed in plastic wrap and exposed to radiography (Kodak, XAR5) for 5 to 6 hours at room temperature or 1 to 2 days at −80°C. DNA fragments (50 ng) were labeled with the standard procedure for random primers DNA labeling system (BRL).

Sequencing analysis

Nucleotide sequences of *Alu*I repetitive DNA fragments were carried out by the dideoxy chain–termination method (Sanger et al., 1977). DNA sequences were analyzed using the Intelligenetics Genalign program at the University of Minnesota Molecular Biology Computer Center, St. Paul, MN.

Foreign-gene preparation and microinjection

Recombinant plasmid pRSV-CAT DNA was digested with *Sma*I in the polycloning sites, and the *Alu*I

fragment from type I or type II repetitive sequences of zebrafish was inserted by blunt-end ligation. Recombinant plasmid DNAs were prepared and purified in quantity with the standard procedure of equilibrium centrifugation in cesium chloride–ethidium bromide gradients (Maniatis et al., 1982). After *Pst*I digestion and phenol/chloroform extraction, the linearized plasmid DNA was then redissolved in ST buffer (88 mM NaCl, 10 mM Tris Cl, pH 7.5) to a final concentration of 50 ng/μl.

Mature male and female zebrafish were separately maintained in aquaria at 25°C ± 1°C under a photoperiod regime of 12 hours of light and 12 hours of dark. Two breeding females and one male were placed overnight in one small chamber at a water temperature of 28°C and under the same photoperiod. Newly fertilized eggs were collected and immediately treated with 0.037% formaldehyde. Treated eggs were microinjected with approximately 2 nl of DNA solution during the first 30 minutes of embryogenesis. Injected eggs developed in Hank's saline solution (137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.25 mM Na_2HPO4, 4.2 mM $NaHCO_3$) until hatching. Developing embryos were individually collected at two different stages (late gastrula and heart beating) into an Eppendorf tube. Each injected embryo sampled was mixed with nine control (late gastrula) embryos to facilitate isolation of genomic DNA.

Acknowledgments

We thank Jeff Essner and two anonymous reviewers for critical reading of the manuscript and Jane Watkins for typing the manuscript. Support for this work came in part from the Minnesota Agricultural Experiment Station, the Minnesota Sea Grant College Program supported by the NOAA Office of Sea Grant, Department of Commerce, under Grant Nos. NOAA-86-AA-D-SG112; Project Nos. RA/5 and RA/7, and NOAA-90AA-SG149; Project Nos. RA/5, RA/6, and RA/7; J.R. 281; the Legislative Commission on Minnesota Resources, The Blandin Foundation, and the Greater Minnesota Corporation.

References

Birnstiel, M., Speirs, J., Purdom, I., et al. (1968). Properties and composition of the isolated ribosomal DNA satellite of *Xenopus laevis*. *Nature* 219:454–463.

Blin, N., and Stafford, D.W. (1976). Isolation of high-molecular-weight DNA. *Nucleic Acids Res* 3:2303–2308.

Borchsenius, S.N., and Chernov, V.M. (1988). *Sau*3A repeat sequences in genomes of two forms of sockeye salmon. *Mol Biol (Moscow)* 22:439–445.

Corneo, G., Ginelli, E., and Polli, E (1967). A satellite DNA isolated from human tissues. *J Mol Biol* 23:619–622.

Darnell, J., Lodish, H., and Baltimore, D. (1986). *Molecular Cell Biology*. N.Y.: Scientific American Books, pp. 427–438.

Davidson, E.H., and Britten, R.J. (1973). Organization, transcription, and regulation in the animal genome. *Q Rev Biol* 48:565–613.

Dretzen, G., Bellard, M., Sassone-Corsi, P., and Chambon, P. (1981). A reliable method for the recovery of DNA fragments from agarose gels and acrylamide gels. *Anal Biochem* 112:295–298.

Fowler, R.F., and Skinner, D.M. (1985). Cryptic satellite rich in inverted repeats comprise 30% of the genome of a hermit crab. *J Biol Chem* 260:1296–1303.

Hallerman, E.M., Schneider, J.F., Gross, M., et al. (1990). Gene expression promoted by the RSV long terminal repeat element in transgenic goldfish. *Anim Biotech* 1:79–93.

Hanahan, D. (1985). *Techniques for Transformation of E. coli*. In: DNA Cloning, vol 1. Glover, D.M. (ed.). pp. 109–135.

Hatch, F.T., and Mazrimas, J.A. (1970). Satellite DNAs in the kangaroo rat. *Biochem Biophys Acta* 224:291–294.

He, L., Li, G., Xie, Y., and Zhu, Z. (1987). Common carp repetitive sequence cloning and characterization. Research Collections, Hubei Province Biochemistry Society, pp. 18–23.

Hew, C.L. (1989). Transgenic fish: present status and future directions. *Fish Physiol Biochem* 7:409–415.

Hinegardner, R. (1968). Evolution of cellular DNA content in teleost fishes. *American Naturalist* 102:517–523.

Houck, C.M., Rinehart, F.P., and Schmid, C.W. (1979). A ubiquitous family of repeated DNA sequences in the human genome. *J Mol Biol* 132:289–306.

Jelinek, W.R., and Schmid, C.W. (1982). Repetitive sequences in eukaryotic DNA and their expression. *Annu Rev Biochem* 51:813–844.

John, B., and Miklos, G.L.G. (1979). Functional aspects of satellite DNA and hetrochromatin. *Int Rev Cytol* 58:1–114.

Kimmel, C.B., and Law, R.D. (1985a). Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. *Dev Biol* 108:75–85.

Kimmel, C.B., and Law, R.D. (1985b). Cell lineage of zebrafish blastomeres. II. Formation of the yolk syncytial layer. *Dev Biol* 108:86–93.

Kimmel, C.B., and Law, R.D. (1985c). Cell lineage of zebrafish blastomeres. III. Clonal analysis of the blastula and gastrula stages. *Dev Biol* 108:94–101.

Kirpichnikov, V.S., Muske, G.A., Scholl-Engberts, A.D., et al. (1990). Genetic structure and allele frequency dynamics in the sockeye salmon population of Lake Dalneye, Kamchatka. *Aquaculture* 84:13–25.

Krayev, A.S., Markusheva, T.V., Kramerov, D.A., et al. (1982). Ubiquitous transposon-like repeats B1 and B2 of the mouse genome: B2 sequencing. *Nucleic Acids Res* 10:7461–7475.

Liu, Z., Moav, B., Faras, A.J., et al. (1990). Development of expression vectors for transgenic fish. *Bio/Technology* 8:1268–1272.

Long, E.D., and Dawid, I.B. (1980). Repeated genes in eukaryotes. *Annu Rev Biochem* 49:727–766.

Maclean, N., and Penman, D. (1990). The application of gene manipulation to aquaculture. *Aquaculture* 85:1–20.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular*

Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Nelson, D.L., Ledbetter, S.A., Corbo, L., et al. (1989). *Alu* polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. *Proc Natl Acad Sci USA* 86:6686–6690.

Page, G.S., Smith, S., and Goodman, H.M. (1981). DNA sequence of the rat growth hormone gene: location of the 5′ terminus of the growth hormone mRNA and identification of an internal transposon-like element. *Nucleic Acids Res* 9:2087–2104.

Powers, D.A. (1989). Fish as model system. *Science* 246:352–358.

Schmid, C.W., and Jelinek, W.R. (1982). The *Alu* family of dispersed repetitive sequences. *Science* 216:1065–1070.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5436–5467.

Singer, M.F. (1982). SINES and LINES: highly repeated short and long interspersed sequences in mammalian genomes. *Cell* 42:433–434.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98:503–517.

Streisinger, G., Walker, C., Dower, N., et al. (1981). Production of clones of homozygous diploid zebrafish (*Brachydanio rerio*). *Nature* 281:293–296.

Stuart, G.W., McMurray, J.V., and Westerfield, M. (1988). Replication, integration, and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* 103:403–412.

Stumph, W., Baez, M., Beattie, W., et al. (1983). Characterization of deoxyribonucleic acid sequences at the 5′ and 3′ borders of the 100 kilobase pair ovalbumin gene domain. *Biochemistry* 22:306–315.

Weiner, A.M., Deininger, P.L., and Efstratiadis, A. (1986). Nonviral retroposons: genes, pseudogenes, and transposable elements generated by the reverse flow of genetic information. *Annu Rev Biochem* 55:631–661.

Zhu, Z., Xu, K., Xie, Y., et al. (1989). A model of transgenic fish. *Sci Sin [B]* 2:147–155.

Molecular Marine Biology and Biotechnology (1992) 1(4/5), 338–345

Selection of promoters for gene transfer into fish

Boaz Moav and Zhanjiang Liu
Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108, U.S.A.

Yonat Groll
Dept. of Zoology, Tel Aviv University, Tel Aviv, Israel 69978.

Perry B. Hackett*
Department of Genetics and Cell Biology, Institute of Human Genetics, 1445 Gortner Ave, University of Minnesota, Minneapolis, MN 55108-1095, U.S.A.

Abstract

A variety of gene constructs containing carp β-actin regulatory sequences were tested for their ability to drive transient expression of the chloramphenicol acetyltransferase reporter gene in 3 fish cell lines: carp epithelial cells (EPC), rainbow trout hepatoma cells (RTH149), and rainbow trout fibroblasts (RTG2). The constructs showed a wide variation in their levels of expression, and there were significant differences in the effects of transcriptional elements in the 3 cell lines. Sequences that enhanced expression in EPC cells were inhibitory in RTH149 and RTG2 cells. All cell lines exhibited the presence of nuclear *trans*-acting factors that could bind to implicated transcriptional control elements. On the basis of the cell culture results, selected constructs were examined for activity in early carp development. Constructs active in embryos and fry were further tested and found to express transgenes in adult fish.

Introduction

Differentiation occurs primarily through regulated transcription of genes to produce the variety of proteins that determine cellular and tissue phenotypes. Understanding of the genetic and molecular mechanisms that activate specific gene transcription requires elucidation of the nature of DNA control sequences in specific genes and the transcription factors that bind those sequences. We initiated an in-depth analysis of the transcriptional control elements of the β-actin genes of carp [*Cyprinus carpio* and *Ctenopharyngodon idella*] using carp β-actin/chloramphenicol acetyltransferase (CAT) fusion genes (Liu et al., 1989, 1990a, 1991). Our studies identified the following 4 transcriptional control regions in the carp β-actin gene: (1) a negative element between 1.1 and 2.2 kb upstream of the transcriptional initiation site; (2) the proximal promoter in the first 100 bp upstream; (3) a negative element close to the 5′ end of intron-1; and (4) an enhancing element near the 3′ end of intron-1. The proximal promter contains 3 transcriptional elements found near many other genes in all classes of vertebrates, the CAAT box, the $CC(A/T)_6GG$ serum response element (or CArG box), and a TATA motif (Liu et al., 1990b). The 3′ intron-1 element has an identical CArG sequence as in the proximal promoter and shows position- and orientation-dependent regulation.

Transcriptional activities of various β-actin/CAT constructs have been examined in zebrafish and goldfish embryos and in tissue-cultured cells (Liu et al., 1990a, 1990c; Moav et al., unpublished observations). Some differences in relative levels of CAT gene expression occur in these different systems. Accordingly, we examined the levels of expression of various constructs in 3 lines of tissue-cultured fish cells—EPC, rainbow trout hepatoma cells (RTH149), and rainbow trout fibroblasts (RTG2)—to determine if heterogeneous tissues respond differentially to produce unique spectrums of CAT activity from assorted β-actin/CAT constructs. The variation in gene expression would presumably be due to a differential assortment of *trans*-acting transcriptional factors in cells of each tissue. We present evidence that cell lines derived from different tissues do direct unequal levels of transcription from constructs with various combinations of β-actin gene regulatory elements. Nevertheless, the EPC cell line

* Correspondence should be sent to this author.

appears to be a good predictor of construct activity in transgenic fish.

Results

CAT constructs

Figure 1 shows the organization of the carp β-actin gene with the identified transcriptional regulatory elements. The first exon does not encode protein sequences, thus allowing the assembly of constructs that contain either only 5′ flanking sequences plus the first 68 bp of exon-1 fused to the CAT reporter gene, or the entire first exon and first intron plus 6 bp of exon-2 fused to the CAT gene. A total of 24 constructs were used in this study (Figure 2).

Constructs 1 to 5 contain different lengths of 5′ flanking sequence; construct 1 has 3,500 bp, construct 2 has 2,300 bp, construct 3 has 1,100 bp (the standard used for most of the constructs containing intron-1), and construct 4 has only the 204 bp proximal promoter. Construct 5 is a control plasmid that has the 3,500-bp 5′ flanking sequence, but it lacks 71 bp of proximal promoter sequence containing the CArG and TATA boxes. Construct 6 has complete exon-1 and intron-1 plus the first 4 bases of exon-2 fused to the CAT gene. Constructs 7 to 18 have modifications within intron-1. Construct 19 lacks all 5′ flanking sequences, including all 3 conserved elements comprising the proximal promoter, but contains 23 bp of the first exon and a complete first intron. Construct 20 has 3,500 bp of 5′ flanking sequences and complete exon-1 and intron-1. Constructs 21 to 24 contain the intron-1 element with the CArG motif repositioned either ahead of or behind the promoter/CAT gene.

These constructs were transfected into EPC, RTH149, and RTG2 cell lines. Forty-eight hours later, cell extracts were made, and the levels of CAT activity were measured for each construct in each cell line (see Figure 2). Figure 3 shows the CAT assay data used to calculate the levels of gene expression in the RTH149 cell line.

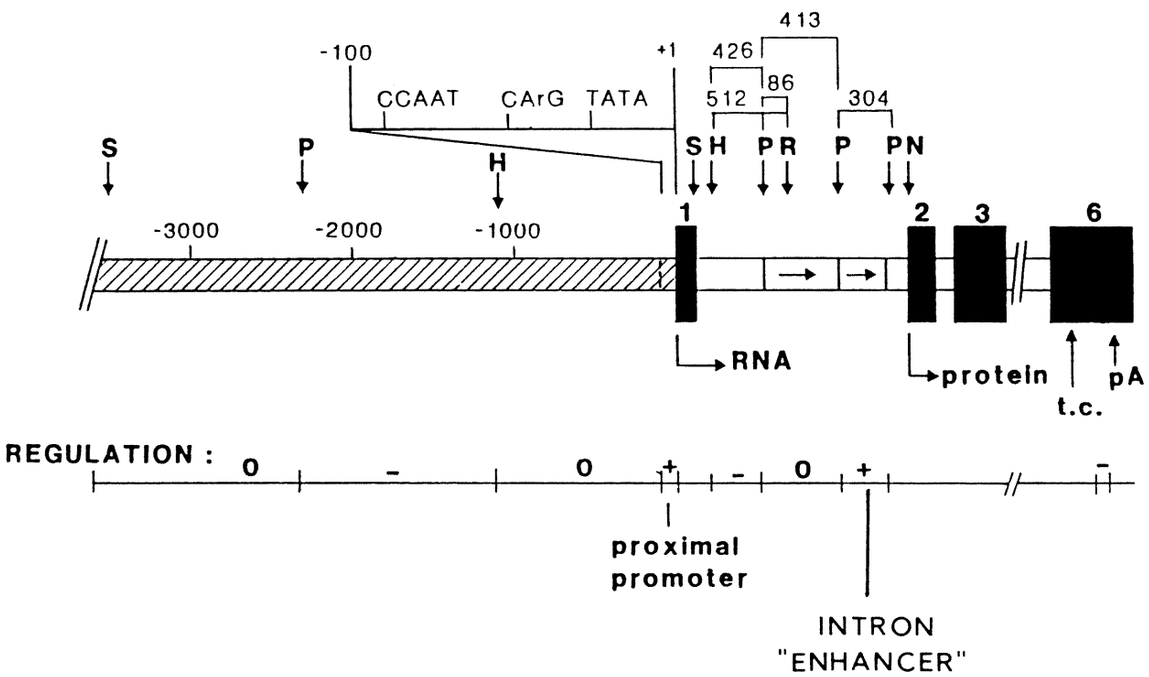


Figure 1. Carp β-actin gene and its identified transcriptional regulatory sites. The 5′ flanking sequence is hatched, and the proximal promoter in the first 100 bp of upstream sequence is expanded to show positions of the CCAAT, CArG, and TATA motifs. The exons of the β-actin gene are dark and numbered, and two regions in the first intron are designated with *arrows* to indicate polarity. Transcriptional and translational initiation *sites* are indicated, as are the sites for translational termination (*t.c.*) and polyadenylation (*pA*). H = *Hin*dIII; P = *Pst*I; N = *Nco*I; R = *Eco*RV; S = *Sst*I restriction endonuclease sites.

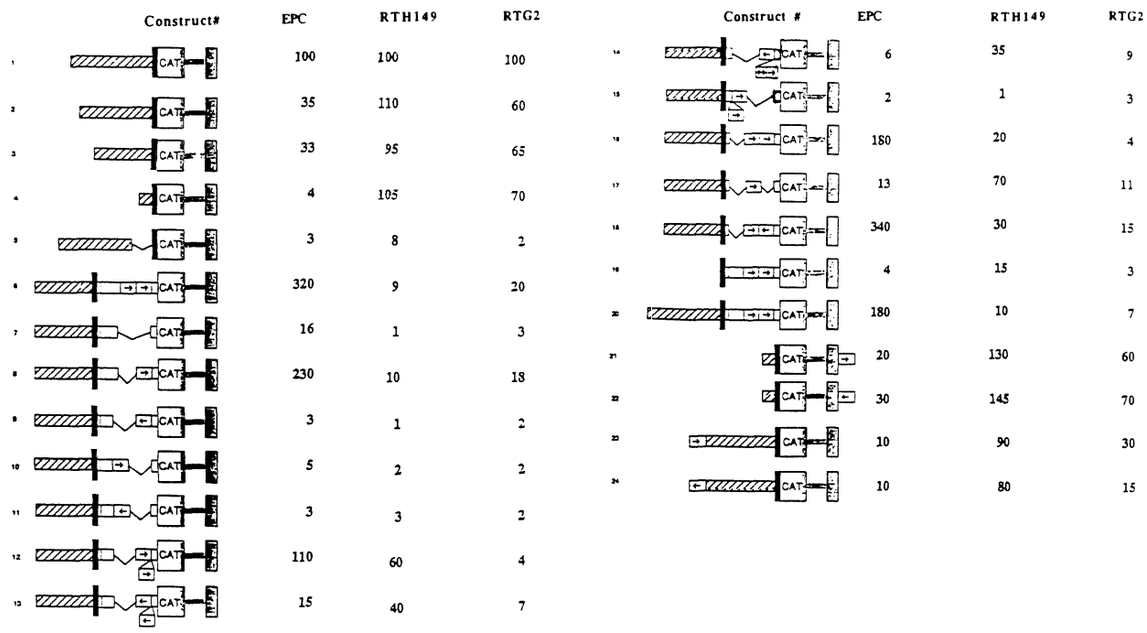


Figure 2. The β-actin/CAT constructs used in this study. The relative values for CAT expression in EPC, RTH149, and RTG2 cell lines are given to the right of each construct. The CAT activity of construct 1 in each cell line has been normalized to 100%. (a) Constructs 1–13; (b) constructs 14–24.

Analysis of the β-actin upstream promoter

Inspection of the CAT activities in extracts containing constructs 1 to 5 (see Figure 2) indicated the following. (1) Maximal levels of expression in RTH149 and RTG2 cells occurred with just 204 bp of 5′ flanking sequence containing the proximal promoter; extensions of the 5′ flanking region did not appreciably alter gene expression. (2) In contrast, the EPC line showed the greatest variation in gene expression, due to a relatively weak proximal promoter and enhancing sequences between −204 and −1,100 (9-fold effect) and between −2,300 and −3,500 (another 3-fold enhancement). (3) The CArG and TATA motifs in the proximal promoter were critical for significant expression, as was evident from the low background levels of CAT activity in all cell lines transfected with construct 5.

Analysis of the transcriptional regulatory sequences in intron-1

The promoterless construct 19 had marginal activity, similar to that obtained with construct 5, as expected. However, transfection of the cell lines with constructs 6 to 20 yielded surprising results. First, intron-1 had only positive effects in the EPC cell line; in the RTH149 and RTG2 cell lines, inclusion of intron-1 generally inhibited expression 30 to 98% and 80 to 98%, respectively. Second, enhanced activity in EPC cell lines required proper orientation of the CArG-containing intron element in all constructs (constructs 6, 8, 12, 16, 20) but one (construct 18). Third, multiple CArG-containing intron-1 elements (constructs 12–14) improved CAT gene expression in RTH149 cells but inhibited activity in EPC and RTG2 cells. Fourth, the data from EPC transfection closely paralleled the results found with mouse L-cell transfection (Liu et al., 1990a), wherein some effects were inconsistent with most other results (e.g., the anomolous activities of constructs 13 and 17 in RTH149 cells and construct 18 in EPC cells).

Position dependence of the intron regulatory element

In the EPC cells, the CArG-containing intron element was generally active in a position-dependent manner when constrained to the intron. To test whether this element could act as a classic enhancer, with position and orientation independence, if placed outside of the intron, the 304-bp intron sequence was positioned behind the CAT

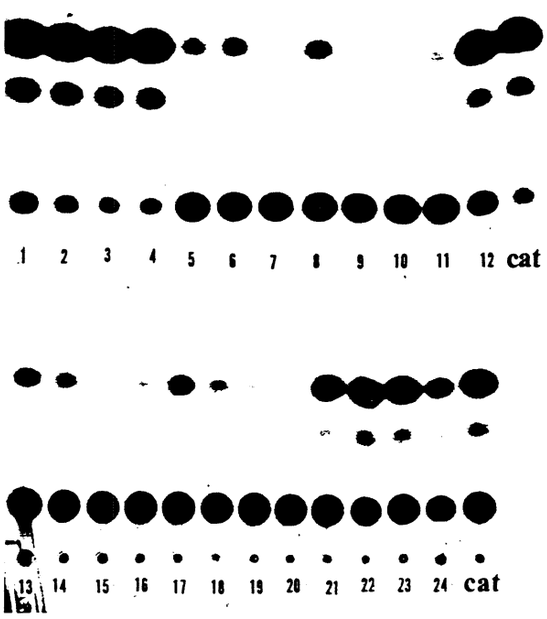


Figure 3. CAT analysis, on silica thin-layer plates, from extracts of RTH149 cells transfected with the different β-actin/CAT constructs. Lane numbers refer to the construct used (see Figure 2); cat-reactions with commercial chloramphenicol acetyltransferase used to standaridize the assays (Liu et al., 1990c) using ^{14}C-labeled chloramphenicol (Cm). The upper spots are acetylated Cm, the lower spots in each lane are the positions of unacetylated Cm.

gene and the poly(A) addition sequence in both orientations (constructs 21 and 22) and was put in both orientations ahead of the 2,300-bp 5′ flanking sequence (constructs 23 and 24). In EPC cells, the intron element was significantly inhibitory in all 4 constructs, acting as a silencer rather than an enhancer of transcription. In contrast, the intron element ahead of the promoter or behind the CAT gene only marginally, if at all, affected CAT expression in the RTH149 and RTG2 cell lines. Clearly, the intron element did not act like a classic enhancer in any of the fish cell lines tested.

Interaction of fish nuclear factors with β-actin regulatory sequences

The mobility shift assay (Dignam et al., 1983; Liu et al., 1991) was used to help define the regions within the regulatory sequences and to investigate the possibility that *trans*-acting factors interacted with each other to promote transcription. Three probes were used: probe 1 contained the 204-bp proximal promoter sequence with the CAAT, CArG, and TATA boxes; probe 2 was the 304-bp intron fragment containing the CArG motif; and probe 3 was a 426-bp fragment from the 5′ end of intron-1 (see Figure 1). Probe 3 has been implicated in some forms of gene regulation (Liu et al., 1990a). Nuclear extracts from all 3 cell lines retarded the mobility of ^{32}P-labeled probe 2 (Figure 4). The ability of 50- and 100-fold excesses of unlabeled probes 2 and 1 to complete partially with the binding suggested specificity of *trans*-acting factors to the previously identified *cis*-acting DNA elements. Although there was not a clear role for the 5′ region of intron-1 in gene regulation (see Figure 2), probe 3 did interact similarly with components from the nuclear extracts from all three cell lines (Figure 4). Thus, despite the vast differences in effects of intron-1 on gene expression in the 3 cell lines, all the cells apparently have factors that can bind to each of the 3 regions in the β-actin gene. These binding studies also supported but did not prove the hypothesis that *trans*-acting factors binding to the proximal promoter and to sequences within the first intron may interact with each other. If so, there must be either alterations of a common factor or additional/alternative *trans*-acting factors in the nuclei of the EPC, RTH149, and RTG2 cells to cause the significantly different levels of expression reported in Figure 2.

Expression of selected promoters during early development in carp

Having identified vectors that were active in fish tissue culture, selected constructs were tested for activity in model fish systems easily maintained in the laboratory (e.g., zebrafish [*Brachydanio rerio*] and goldfish [*Carrasius auratus*]). The activity of the constructs in zebrafish paralleled that found in EPC cells, whereas the intron elements neither enhanced (as seen in EPC cells) nor inhibited (as in the RT cell lines) expression of the transgene in goldfish (Moav et al., unpublished observations). Consequently, several of the promising β-actin vectors that showed transcriptional strength in the zebrafish and goldfish were tested for activity in larger fish of economic importance. Some results demonstrating early expression of the "all-fish" β-actin expression vectors (Liu et al., 1990c) in northern pike and walleye have been reported (Moav et al., 1992).

We extended these studies to analyze the activity of the vectors in young and adult carp. Four of the constructs were microinjected into fertilized eggs, and CAT expression was monitored in developing

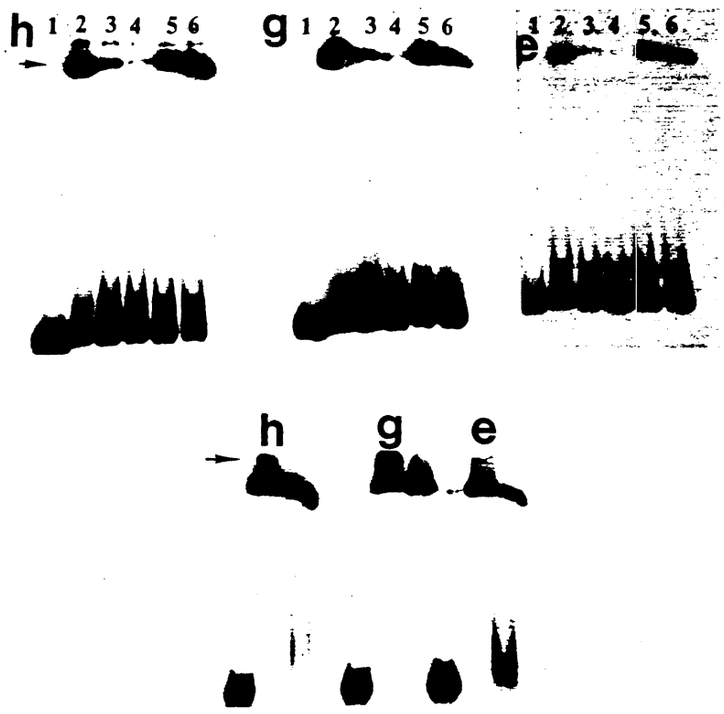


Figure 4. Sequence-specific binding of factors to intron elements revealed by mobility shift assay as described in Materials and Methods. Nuclear extracts: h = RTH149 cell line; g = RTG2 cell line; e = EPC cell line. The *arrows* show the position of shifted DNA fragments with bound nuclear factors. (Top) Mobility shift assay of probe-2, the 304-bp *PstI* fragment from the 3′ end of intron-1 (see Figure 1). Lane 1: no nuclear extract; lanes 2–6: assays contain 10 μg nuclear extracts. Lane 2: no specific competitor; lanes 3 and 4: 25-fold and 50-fold excesses of probe-2; lanes 5 and 6: 25-fold and 50-fold excesses of probe-1. (Bottom) Mobility shift assay of probe-3, the 426-bp *Hin*dIII/*Pst*I fragment from the 5′ end of intron-1 (see Figure 1). *Left* lanes in each group of assays were from reactions lacking nuclear extract and specific competitor DNA. *Middle* and *right* lanes have nuclear extract; right lanes also contain 50-fold excesses of probe-3 competitor DNA.

embryos and fry. Figure 5 shows the CAT activity from construct 4 with the proximal promoter (medium expression); constructs 6 and 8 with the proximal promoter plus intron-1, with the CArG element in the proper orientation (high expression); and construct 9, with an inverted CArG element (low activity). Both constructs 6 (GH3′) (which have the 3′ end of the salmon growth hormone gene rather than the 3′ end of the SV40 early genes; designated FV-2 in Liu et al., 1990c) and 8 were active in developing carp zygotes and fry. The results with constructs 6 (GH3′), 8, and 9 emphasize the conclusion that inclusion of all of intron-1 is not important; rather, retaining the CArG element in its proper orientation is apparently the most important feature, as suggested by the data obtained with EPC cells (compare Figures 2 and 5).

On the basis of the results in Figure 5, transgenic carp were prepared by comicroinjection of two constructs, the β-actin/salmon growth hormone cDNA (Hew et al., 1989; Liu et al., 1990c; Moav et al., 1992) and either construct 6 (GH3′) or 8, which had shown the highest activities in early development. Table 1 demonstrates that (1) both constructs were nearly equally active in adult transgenic carp, and (2) the fins were the best tissue for determining expression of the transgene because expression per microgram of extract had the highest activity and the samples were of adequate size. In these studies on promoter activities in adult carp, the chromosomal integration status of the construct was not examined.

Expression in EPC cells is useful for screening putative expression vectors

Our results show that different piscine cell lines derived from specific tissues exhibit individual expression patterns for a variety of expression vectors containing different transcriptional control elements of the carp β-actin gene. This conclusion suggests that the cell lines contain an assortment of *trans*-acting factors that regulate expression from the assorted constructs. Moreover, expression in EPC cells appears to be a good predictor of expression in transgenic fish. The results also demonstrate that researchers have a variety of vectors, some of which may have specific expression capabilities in various differentiated tissues in fish. In addition, these vectors may be useful for investigations of the molecular biology of gene expression during fish growth and development.

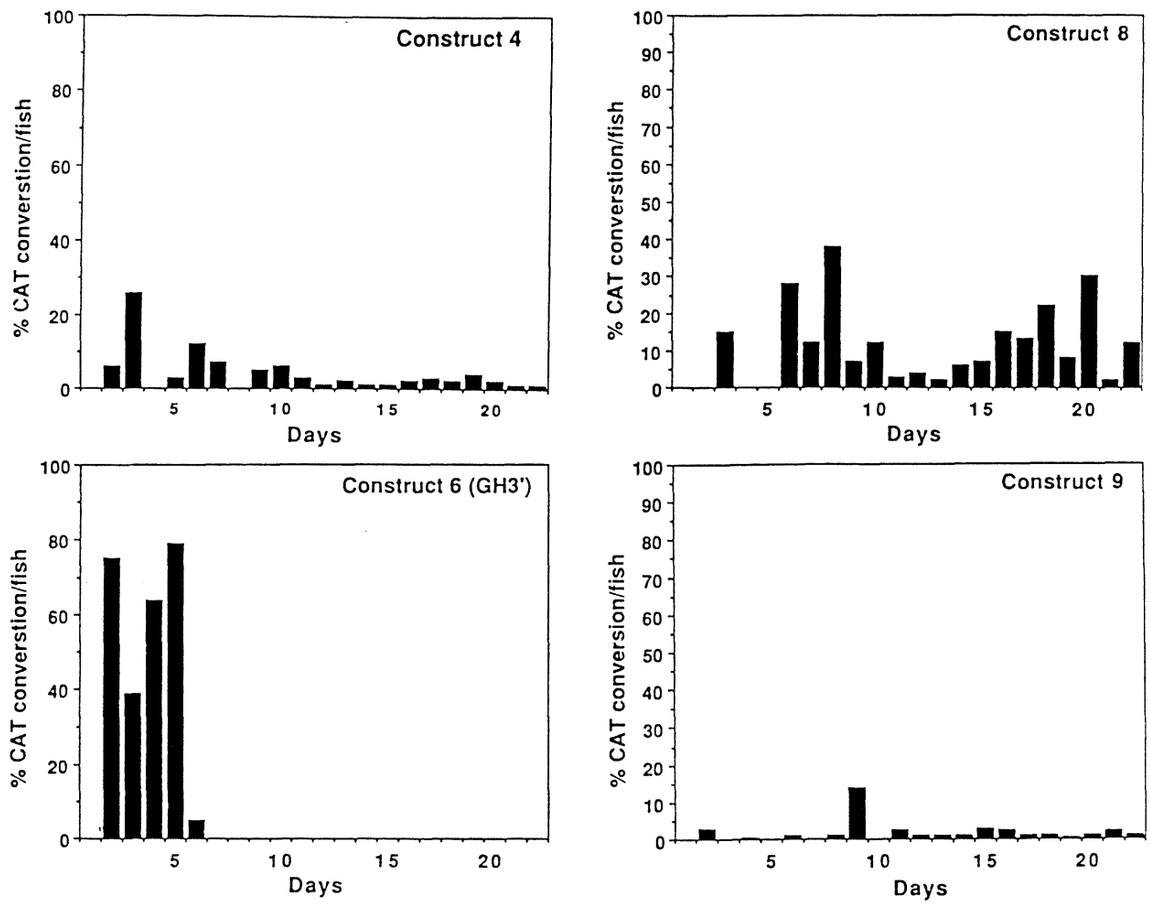


Figure 5. Expression of β-actin/CAT constructs during early development in carp. Five to 10 embryos or fry were pooled each day after fertilization/microinjection (see Materials and Methods) for analysis of CAT activity. The equivalent of one embryo extract was taken from the pooled extracts and analyzed (see Figure 3). Construct numbers are given in the upper corner of each panel. Constructs 4, 8, and 9 are shown in Figure 2. Construct 6 (GH3') is identical to construct 6 shown in Figure 2, except that it contains the 3′ end of the chinook salmon growth hormone gene rather than the 3′ end of the SV40 early gene region.

Materials and Methods

Construction of plasmids

Construction of the 24 principal plasmids used in these experiments has been previously described (Liu et al., 1990a). Essentially, these constructs consist of the carp β-actin gene promoter/enhancer sequences juxtaposed to the bacterial CAT gene with the SV40 early region intron and polyadenylation sequences. In the study of transgene expression in the carp, a modification of construct 6 was made such that the SV40 intron/poly(A) addition/cleavage signal was replaced with the 3′ end of the salmon growth hormone gene (Hew et al., 1989) as described by Liu et al. (1990c). Construction of the FV-2 plasmid (Liu et al., 1990c) harboring the salmon GH gene has been described (Gross et al., 1992).

DNA transfection of cultured fish cell lines and CAT assay

The EPC cell line (Fijan et al., 1983) was obtained from Dr Daniel Chourrout (INRA, France). The rainbow trout hepatoma (RTH149; ATCC CRL1716) and rainbow trout gonad (RTG2; ATCC CCL55) cell lines were obtained from the ATCC. DNA transfection of the 3 fish cell lines was accomplished using the basic $CaPO_4$ method of Graham and Van der Eb

Table 1. Expression of selected β-actin/CAT constructs in adult carp. Two transgenic carp that showed positive CAT fin clips were sacrificed for analysis of CAT gene expression in different tissues. Control samples were taken from sibling fish that were not microinjected with DNA.

Construct No.	Age	Tissue	CAT Expression[a]
6	7 mo	Head	0.014
		Muscle	0.019
		Fin	5.9
8	12 mo	Head	0.042
		Muscle	0.019
		Fin	4.5
Controls[b]	7 mo	Fin	0.46
	7 mo	Fin	0.97
	7 mo	Fin	0.88
	12 mo	Fin	0.37
	12 mo	Fin	0.76
	12 mo	Fin	0.74

[a] Percent CAT conversion per μg extract.

[b] From uninjected fish.

(1973) with some modifications (Liu et al., 1990c). CAT activities were assayed by the procedure described by Gorman and colleagues (1982) with slight modifications (Liu et al., 1990a). All assays were compared with activities of control CAT enzyme obtained from Sigma Chemical Co. All autoradiograms were developed in their linear range for accurate quantification.

Microinjection of carp eggs and culture of carp zygotes

Male carp (*Cyprinus carpio*) and female carp (Japanese ornamental Koi carp) were induced to spawn by injection of pituitary extract. Eggs and sperm were obtained by standard stripping procedures and kept at 4°C. One hundred eggs were mixed with sperm and activated by well-water in a tissue culture dish. Excess sperm were removed after 1 minute, and the eggs that adhered to the plastic were washed several times with well-water at room temperature. Microinjection of the zygotes was performed at room temperature in Holtfreter's solution (3.5 gm NaCl, 0.05 KCl, 0.1 gm CaCl, 0.2 gm $NaHCO_3$ in 1,000 mL water). Microinjection by air pressure–controlled PL1-100 Pico-Injector apparatus (Medical Systems Co.) was performed within 5 hours after spawning and within 20 minutes of fertilization of each batch of eggs. Approximately 20 to 40 nL, equivalent to 50 to 250 pg, of DNA solution was microinjected into each egg. Following microinjection, the carp zygotes were washed several times with Holtfreter's solution at room temperature and then incubated at 26°C in well-water. Hatching under these conditions occurs 48 to 50 hours after fertilization.

Mobility shift assays

Nuclear extracts from rainbow trout RTH149 and RTG2 cells and carp EPC cells were prepared as described by Dignam and associates (1983) with minor modifications (Liu et al., 1991). The nuclear extract was dialyzed for 5 hours against 100 vol of buffer (20 mmol/L HEPES (pH, 7.0), 20% (v/v) glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT) containing the protease inhibitors PMSF, pepstatin, and leupeptin. Five to 10 μg of the dialyzed nuclear extract was added to 20-μL reactions containing 2 μg pUC119 DNA and 2 μg poly(dI/dC) as nonspecific competitors, 0.1 to 1.0 ng of 3′ end-labeled probe, and appropriate specific competitor DNA. After incubation at room temperature for 10 minutes, the reaction mixtures were loaded onto 6% (29:1) polyacrylamide:bisacrylamide gels.

Acknowledgments

This work was sponsored by BARD grant US-1517-88 (B.M.), Public Health Service grant RR-06625 from the National Institutes of Health, the Legislative Committee for Minnesota Resources, and the National Oceanographic and Atmospheric Administration, Office of Sea Grant under grant NOAA-86AA-D-SG112 R/A-7 (paper JR-286). Z.L. was supported by a stipend from Minnesota Sea Grant.

We thank Drs S. Rothbard (YAFIT and the Fish Breeding Institute, Gan-Shmuel, Israel), G. Hulata, and N. Cherfas (Dor) for providing carp eggs. We thank J. Essner for reading the manuscript.

References

Dignam, J.D., Lebowitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiated by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 11:1475–1489.

Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., and de Kinkelin, P. (1983). Some properties of the *epithelioma papulo-*

sum cyprini (EPC) cell line from carp *Cyprinus carpio*. *Ann Virol* 134E:207–220.

Gorman, C.M., Moffat, L.F., and Howard, B. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* 2:1044–1051.

Graham, F.L., and Van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467.

Gross, M.L., Schneider, J.F., Moav, N., Moav, B., Alvarez, C., Myster, S.H., Liu, Z., Hallerman, E.M., Hackett, P.B., Guise, K.S., Faras, A.J., and Kapuscinski, A.R. (1992). Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes. *Aquaculture* 103:253–273.

Hew, C.L., Trinh, K.Y., Du, S.J., and Song, S. (1989). Molecular cloning and expression of salmon pituitary hormones. *Fish Physiol Biochem* 7:375–380.

Liu, Z., Zhu, Z., Roberg, K., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1989). The β-actin gene of carp (*Ctenopharyngodon idella*). *Nucleic Acids Res* 17:5850.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1990a). Functional analysis of elements affecting expression of the β-actin gene of carp. *Mol Cell Biol* 10:3432–3440.

Liu, Z., Zhu, Z., Roberg, K., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1990b). Isolation and characterization of the β-actin gene of carp (*Cyprinus carpio*). *DNA Seq J DNA Seq Map* 1:125–136.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1990c). Development of vectors for transgene expression in fish. *Biotechnology* 8:1268–1272.

Liu, Z., Moav, B., Faras, A.J., et al. (1991). Importance of the CArG box in regulation of β-actin-encoding genes. *Gene* 108:211–217.

Moav, B., Liu, Z., Moav, N.L., Gross, M.L., Kapuscinski, A., Faras, A., Guise, K., and Hackett, P. (1992). Expression of heterologous genes in transgenic fish. In: *Transgenic Fish*. Hew, C.L., (ed.). Singapore: World Scientific Publishing Co, pp. 120–141.

Transgenic Research **2**, 153–161 (1993)

Regulation of expression of transgenes in developing fish

BOAZ MOAV[1,2], ZHANJIANG LIU[2], LJUBICA D. CALDOVIC[2], MARK L. GROSS[3], ANTHONY J. FARAS[3,4] and PERRY B. HACKETT[2,4]*

[1]*Department of Zoology, Tel Aviv University, Tel Aviv, Israel*
[2]*Department of Genetics and Cell Biology, 1445 Gortner Avenue, University of Minnesota, St. Paul, MN 55108-1095, USA*
[3]*Department of Microbiology and* [4]*Institute of Human Genetics, University of Minnesota, Minneapolis, MN 55455, USA (Fax: +1 612 625 5754)*

Received 11 September 1992; revised 27 October 1992; accepted 11 November 1992

The transcriptional regulatory elements of the β-actin gene of carp (*Cyprinus carpio*) have been examined in zebrafish and goldfish harbouring transgenes. The high sequence conservation of the putative regulatory elements in the β-actin genes of animals suggested that their function would be conserved, so that transgenic constructs with the same transcriptional control elements would promote similar levels of transgene expression in different species of transgenic animals. To test this assumption, we analysed the temporal expression of a reporter gene under the control of transcriptional control sequences from the carp β-actin gene in zebrafish (*Brachydanio rerio*) and goldfish (*Carrasius auratus*). Our results indicated that, contrary to expectations, combinations of different transcriptional control elements affected the level, duration, and onset of gene expression differently in developing zebrafish and goldfish. The major differences in expression of β-actin/CAT (chloramphenicol acetyltransferase) constructs in zebrafish and goldfish were: (1) overall expression was almost 100–fold higher in goldfish than in zebrafish embryos, (2) the first intron had an enchancing effect on gene expression in zebrafish but not in goldfish, and (3) the serum-responsive/CArG-containing regulatory element in the proximal promoter was not always required for maximal CAT activity in goldfish, but was required in zebrafish. These results suggest that in the zebrafish, but not in the goldfish, there may be interactions between motifs in the proximal promoter and the first intron which appear to be required for maximal enhancement of transcription.

Keywords: β-actin gene; goldfish; promoter; transcription; zebrafish

Introduction

Complex spatial and temporal regulation of gene expression in multicellular organisms are required for both proper development and homeostasis. This is achieved primarily at the level of transcription, involving activators, repressors and squelchers of RNA synthesis (Ptashne, 1988). Tissue-cultured cells are insufficient for identifying and characterizing the transcriptional regulatory sequences responsible for differential gene expression that occurs in multiple differentiated tissues of developing and adult organisms. To circumvent this problem, transgenic organisms can be used to characterize tissue- and developmental-specific transcriptional control elements. For many reasons, fish are being recognized as an excellent model system for investigations of developmental genetics (Kimmel, 1989; Powers, 1989; Rossant and Hopkins, 1992) and cancer genetics (Schwab, 1987; Schartl *et al.*, 1990). Firstly, most fish embryos develop outside the mother, allowing easy inspection and access to the developing embryo. Secondly, fish eggs are easy to obtain in large quantities without any trauma or injury to the female and, in some species, such as the zebrafish, may be produced daily. Thirdly, the embryos are fairly large and hardy, simplifying their handling. Several groups have microinjected genes into fish zygotes for a variety of

*To whom correspondence should be addressed.

purposes including development of growth-enhanced fish (reviewed by Fletcher and Davies, 1991; Hackett, 1992). Early experiments employed promoter elements from mammals, birds and viruses. More recently, promoter elements isolated from piscine species have been tested (Liu *et al.*, 1990a,b,c; Friedenreich and Schartl, 1990; Gong *et al.*, 1991; Shears *et al.*, 1991; Winkler *et al.*, 1991; Moav *et al.*, 1992a,b; Du *et al.*, 1992; Xiong *et al.*, 1992). All of these studies demonstrated that transcriptional control sequences from mammals, birds and fish are able to direct RNA synthesis in vertebrate cells.

We (Liu *et al.*, 1990c; Moav *et al.*, 1992a,b) have constructed expression vectors that employ the regulatory elements of the well-characterized carp β-actin gene (Liu *et al.*, 1990a,b, 1991; Moav *et al.*, 1992b). These studies showed that the organization of the β-actin gene of carp is similar to that found in land-vertebrate genomes, in which the first exon is non-coding and transcriptional control elements reside in the first intron. Like β-actin genes in other higher eukaryotes, fish β-actin genes have a serum-responsive element, the $CC(A/T)_6GG$ (CArG) box, which is evolutionarily conserved in actin and other serum-responsive genes (Minty and Kedes, 1986; Boxer *et al.*, 1989; Orita *et al.*, 1989; Subramaniam *et al.*, 1989; Walsh, 1989; Liu *et al.*, 1990a).

The high sequence conservation of the putative regulatory elements in the β-actin genes of animals suggests that their function is also highly conserved. We assumed that transgenic constructs with similar transcriptional control elements would promote similar levels of transgene expression, allowing the elements to be used in the construction of expression vectors for transgenic animal studies. To test this assumption, we analysed the temporal expression of a reporter gene under the control of transcriptional control sequences from the carp (*Cyprinus carpio*) β-actin gene in two species of fish used as model systems; the zebrafish (*Brachydanio rerio*) and the goldfish (*Carrasius auratus*). Our results indicated that, contrary to expectations, some combinations of transcriptional control elements affected the level of duration of gene expression differently in developing zebrafish and goldfish.

Materials and methods

Recombinant constructs

The constructs used have been described previously (Liu *et al.*, 1990b, 1991). Essentially, all the tested constructs contain portions of the carp β-actin gene ligated to the bacterial chloramphenicol acetyltransferase (CAT) gene to which either the 3′-end of the SV40 early region with its intron and poly(A) sequence (Gorman *et al.*, 1982) or the 3′-end of the chinook salmon growth hormone gene (Hew *et al.*, 1989) was juxtaposed as described previously (Liu *et al.*, 1990c).

Collection of fish eggs

Zebrafish (*Brachydanio rerio*) eggs were harvested within 1 h after fertilization. Essentially, two females and one male were kept per breeding box in an aquarium with a controlled photoperiod. The procedures recommended in *The Zebrafish Book* (Westerfield, 1989) were used to induce and enhance mating. To obtain newly fertilized eggs, a funnel with tygon tubing at the bottom was placed underneath the breeding box to allow easy and gentle collection by siphoning aquarium water with eggs into a beaker. Zygotes were washed with 10% Hank's saline (13.7 mM NaCl, 0.54 mM KCl, 0.13 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.025 mM Na_2HPO_4, 0.42 mM $NaHCO_3$) and placed in Petri dishes with a 2 mm layer of 1.5% agarose. The eggs were conveniently transferred with large-mouth Pasteur pipettes. Most of the solution was removed leave the zygotes barely covered; this procedure prevents drying and enhances the adhesion of the zygotes to the plate during the microinjection. Collection of goldfish (*Carrasius auratus*) sperm and eggs from males and females was done by standard stripping procedures; sperm and eggs were kept at 4° C until use. One hundred eggs were mixed with sperm and activated by well water on a Petri dish. Water and excess sperm were removed after 1 min and the eggs that adhered to the plastic were washed several times with well water at room temperature and placed in Holtfreter's solution (3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$, in 1 L) (Holtfreter, 1931) for microinjection.

Microinjection of the zygotes

Microinjection was performed at room temperature with a mechanical micromanipulator, using an air-pressure-controlled PLI-100 Pico-Injector apparatus (Medical Systems Co., Greenvale, NY, USA) and microcapillaries with 1-5μm tip diameters. Appoximately 4–20 nl (50–250 pg) of DNA, in solution stained with a drop of food dye (Schilling), was microinjected into each egg. After microinjection (about 10 eggs per min) the eggs were washed in Hank's solution and placed in 250-ml glass beakers (50–100 eggs per beaker) and kept at 28° C in a water bath. Five hours after microinjection, the embryos were treated for 15 min with 0.1% formaldehyde in Hank's solution to prevent fungal infection.

Culture of zebrafish and goldfish zygotes

Following fertilization and microinjection, the zebrafish embryos were washed several times a day until hatching. After hatching, the young zebrafish were fed with live infusoria (*Little Fry Formula*, Jungle Labs Co., Cibolo, TX, USA) for 2 weeks and then transferred to regular aquaria at 28° C for further growth. After 10 h in the Holtfreter's solution following formaldehyde treatment, the goldfish embryos were placed in 0.5× Holtfreter's solution for 12 h and then washed and kept in well water

until hatching (about 4–5 days at 20°–22° C). Fry were reared in net cages submerged in an aquarium at 20° C.

CAT assays

CAT assays were done on 10–20 embryos, which were suspended in 1 ml of 0.25 M Tris (pH 7.5) by homogenization with a Polytron Homogenizer for 30 s at room temperature followed by three cycles of freezing and thawing. The homogenate was centrifuged at 15000 × **g** for 10 min at 4° C to pellet cellular debris. The supernatant was used for assay of CAT activity at 37° C for 1 h with [^{14}C] chloramphenicol (Amersham, Arlington Heights, IL, USA) and acetyl-coenzyme-A (Sigma, St. Louis, MO, USA) (Gorman *et al.*, 1982). In order to get a quantitative measure of transgene activity in early development, we had to take into consideration the non-earity of CAT conversion percentages. The original ta of percentage conversion of [^{14}C]-labelled chloramphenicol to acetylated chloramphenicol was corrected using a standard curve. The standard curve was obtained by adding purified CAT enzyme (Sigma, St. Louis, MO, USA), at concentrations ranging from 1 to 75 units, to our standard assay and measuring the resultant chloramphenicol acetylation. The CAT activity values presented in Figs 2, 3 and 4, and in Table 1 indicate corrected CAT enzyme activity per zygote. Repeated assays of CAT activity in triplicate samples, taken from single pools of embryonic lysates, varied by 5 to 10% (Z. Liu and B. Moav, unpublished).

DNA analysis

DNA was extracted and analysed by dot-blotting, using uniformly [^{32}P]-labelled CAT gene as a probe, according to Sambrook *et al.* (1989). Twenty embryos were taken at 12 h and 15 embryos were pooled at 2, 4, 6, 8 and 10 days after microinjection, and pooled for DNA extraction and analysis.

sults

Regulatory sequences for transcriptional units in vertebrates generally reside in three locations: 1) the proximal promoter within the first 100 nucleotide pairs preceding the transcriptional initiation site; 2) distal sequences, sometimes extending several kilobases, upstream of the proximal promoter; and 3) regulatory regions behind the promoter, often in introns (Mitchell and Tjian, 1989). Accordingly, we analysed the transcriptional regulatory regions of the carp β-actin gene by linking these sequences to the bacterial CAT reporter gene (Gorman *et al.*, 1982; Liu *et al.*, 1990b) and examining resultant CAT activities in zygotes that developed from fish eggs microinjected with the various constructs. The constructs used for this investigation are shown in Fig. 1; all included the first non-coding exon of the β-actin gene. These four constructs were selected from the 28 constructs that have been examined in tissue-cultured mammalian and piscine cells (Liu *et al.*, 1990b, 1991; Moav, 1992a,b). Construct 1 has only the proximal promoter in 204–bp of 5′ flanking sequence. Construct 2 has, in addition to the sequences in construct 1, the remainder of exon-1 plus the complete first intron and the leading portion of exon-2 that contains the 3′-splice site. Constructs 3 and 4 lack the conserved CArG box in their proximal promoters but are otherwise identical to constructs 1 and 2. These constructs were microinjected into either zebrafish or goldfish within 1 h after fertilization and gene expression was examined over the following 7–10 days. The larger goldfish produce many more eggs per female and zygotes hatch about 4 to 5 days after fertilization at 20° C–22° C, compared to zebrafish which hatch at about 3 days post-fertilization at 28° C. Supercoiled constructs were injected into the zygotes to reduce complications due to differential rates of integration, sites of integration, and other modifications of the transgenic DNA that could occur with linearized constructs (Moav *et al.*, 1992a; Liu *et al*, 1991).

Transgene expression in zebrafish and goldfish

The necessity of an intact β-actin proximal promoter was shown in several cell lines (Liu *et al.*, 1990b,c; Moav, 1992a,b). For analysis of transgene expression in zebrafish, batches of several hundred zygotes were microinjected with each construct. From these, 15–20 embryos or hatchlings were randomly selected for each assay of CAT gene activity. The data in Fig. 2 demonstrated that an intact 204-bp proximal promoter consisting of a TATA box, a CArG box, and a CAAT box (Fig. 1) was sufficient to drive expression of the CAT reporter gene in zygotes of both fish species but the level of expression in goldfish zygotes was more than 100-fold higher per embryo than in zebrafish. In both species the onset of CAT activity was around day 4.

We previously found that the first intron of the carp β-actin gene enhanced downstream gene expression five-fold in mouse cells (Liu *et al.*, 1990b), presumably owing to interactions between factors binding to sequences in the proximal promoter and in the first intron (Kawamoto *et al.*, 1988; Ng *et al.*, 1989; Liu *et al.*, 1991). The effects of the intron on CAT gene expression were tested in microinjected zebrafish and goldfish eggs. The addition of intro-1 to the promoter resulted in a 5-fold increase in CAT activity in zebrafish (Fig. 3A) but only a modest increase in the goldfish zygotes (Fig. 3B). In these experiments, as well as those that follow, we did not examine mRNA levels directly; we have previously shown that CAT expression from these β-actin/CAT constructs was proportional to steady-state message levels and that neither splicing nor stability was noticeably affected by the juxtapositioning of the different elements in our constructs (Liu *et al.*, 1990b). As shown in Figs. 2B and 3B,

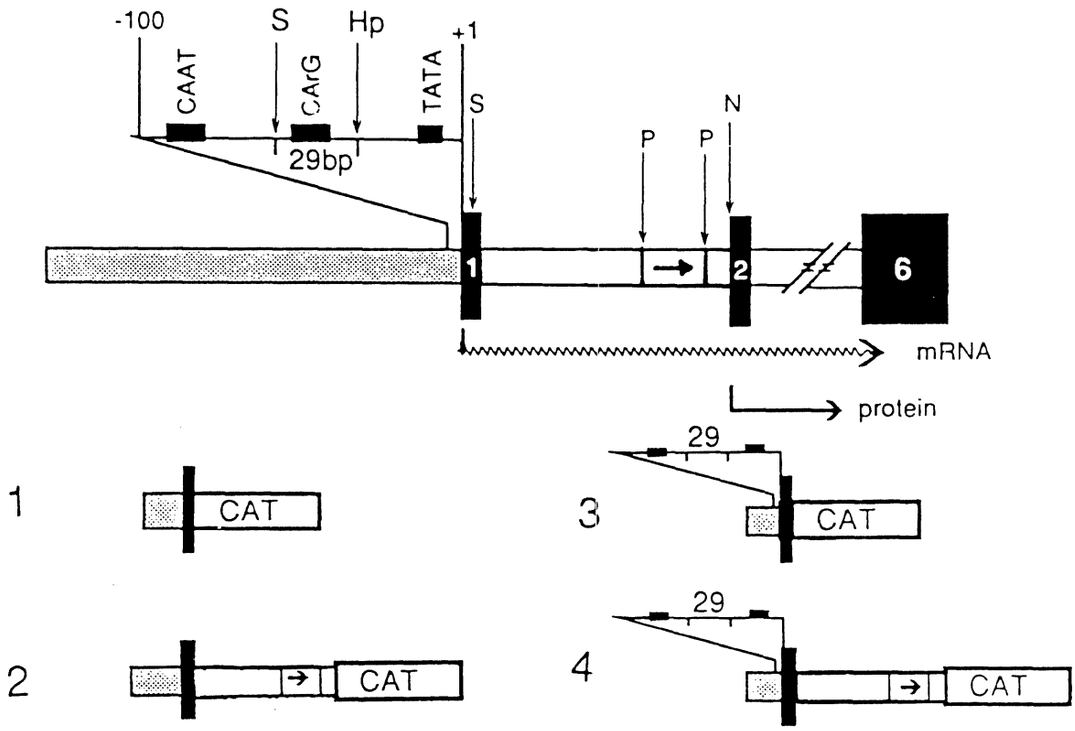


Fig. 1. Schematic diagram of constructs used in this study. Top: schematic of the carp β-actin gene with 5′-flanking sequences (shaded region), expanded 100-bp sequence in the proximal promoter with the CAAT, CArG and TATA boxes shown, exons 1, 2, and 6 (blackened regions), and introns (unshaded) showing regions of intron-1 that contain conserved motifs (arrows designate orientation). The transcriptional (+1) and translational initiation sites are indicated. S = *Sst* I; Hp = *Hpa* II; P = *Pst* I; N = *Nco* I; restriction endonuclease sites. Bottom: the constructs are shown with 5′-flanking sequences which are not to scale; constructs 1–4 have the 204–bp proximal promoter. In constructs 3 and 4, a 29–bp sequence containing the CArG box in the proximal promoter was replaced by a 29–bp linker. The region in the intron flanked by *Pst* I sites contains a copy of the CArG motif. Behind the CAT gene were either the SV40 3′ sequences from pSV40 or the salmon growth hormone gene 3′-end (not shown in the diagrams, see Liu *et al.*, 1990c).

constructs 1′ and 2′, substitution of the Atlantic salmon growth hormone polyadenylation signal for the comparable sequences from SV40 did not appreciably affect expression. The activities of these constructs are shown since they contain only piscine transcriptional regulatory sequences, which is important when these constructs are used as expression vectors (Liu *et al.*, 1990c). CAT gene expression over days 3–8 post-fertilization was calculated and the total activity integrated for each microinjection experiment to facilitate comparison of the different transgenic constructs. Table 1 shows the CAT activity values for the data in Figs 2 to 4 as well as results obtained previously from tissue-cultured mouse cells for comparison.

A single CArG box is sufficient for high-level gene expression in goldfish

The conserved CArG motif was required for expression of actin genes in tissue culture (Chow and Schwartz 1990; Liu *et al.*, 1991). However, requirements for conserved motifs for expression may differ in tissue culture and in transgenic animals (Pinkert *et al.*, 1987; Tronche *et al.*, 1989; Swift *et al.*, 1989). We tested the requirement f the CArG box in the proximal promoter during zebrafi.. development, using constructs 3 and 4 which lack the CArG motif but maintain the normal spacing between the CAAT and TATA boxes. In constructs with just the proximal promoter, precise replacement of the CArG element in a 29 bp region lowered CAT activity 2-fold in zebrafish and 100-fold in goldfish relative to construct 1 (Fig 4A,B). When constructs which included the first intron, but lacked the CArG box in the promoter, were injected in developing zebrafish zygotes, expression was reduced 20-fold relative to that from construct 2 (Fig. 4A). In distinct contrast, removal of the CArG motif from the proximal promoter did not impair, and possibly may have enhanced, transcription from the intron-containing construct in goldfish (Fig. 4B and Table 1). The reductions in CAT gene expression suggest a positive activation role of the CArG element in the proximal promoter

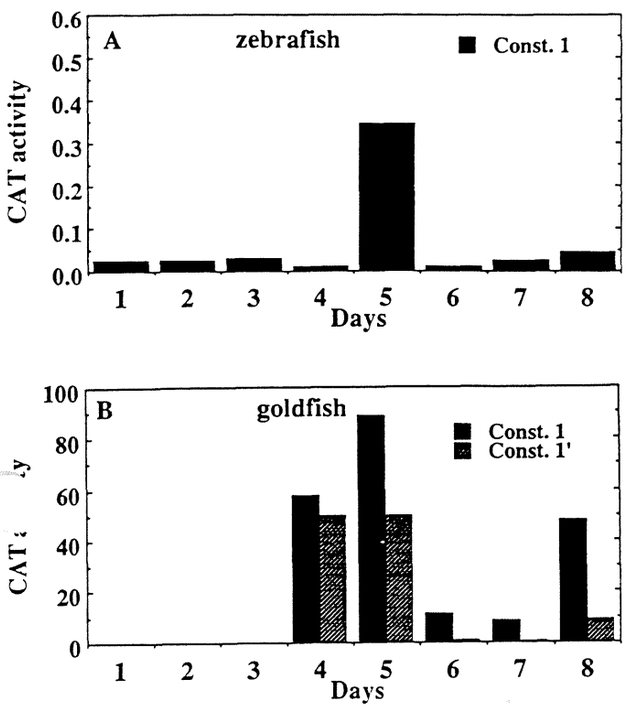


Fig. 2. CAT activity per embryo or hatchling from carp β-actin/CAT constructs in zebrafish and goldfish. Microinjected zygotes were maintained as described (Materials and methods). 10-20 embryos or hatchlings were pooled on various days post-fertilization/microinjection for analysis of CAT activity. The equivalent of one embryo extract (5-10% of the pooled extract) was analysed by thin layer chromotography on silica gels as described (Materials and methods). Construct designations (Fig. 1) are given in the upper right corner of each panel. Two constructs are shown in goldfish; the prime on construct 1′ indicates that the salmon growth hormone 3′-region replaced the SV40 3′-region. Note the difference in scale for levels of CAT activity for constructs in zebrafish and goldfish.

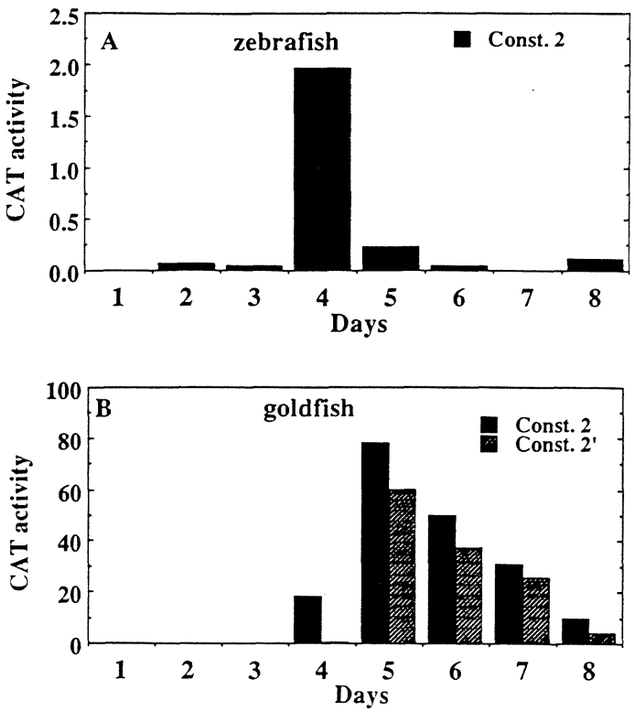


Fig. 3. CAT activity per embryo or hatchling from constructs with intron elements microinjected into embryos as described in Fig. 2. Construct designations (Fig. 1) are given in the upper right corner of each panel. Two constructs are shown in goldfish; the prime on construct 2′ indicates that the salmon growth hormone 3′-region replaced the SV40 3′-region.

uuring zebrafish development, whereas only a single CArG box in either the proximal promoter or the first intron is required during goldfish development.

Rates of transgene loss after microinjection

In every experiment, expression of the different β-actin promoter/CAT constructs decreased after peaking between days 4 and 7. In several experiments where expression was followed for 14 or more days, CAT activities in the embryos dropped to levels near to background although in some cases at later times the CAT activities rose a little (Moav *et al.*, 1992a and unpublished results). The decrease in CAT activity may have been due to a reduction in transcription of the transgenes, disappearance of the constructs, or both. Accordingly, we examined the persistence of the transgenes over the first ten days of zebrafish development by microinjecting approximately 5×10^5 copies of different constructs into embryos. Over the four days following microinjection, the zygotes lost about 50–70% of these constructs. A further 50–90% reduction in the number of constructs/embryo occurred during the next four days (Fig. 5 and Table 2). The persistence of five different constructs was tested in this way; all showed approximately the equivalent stability (L. Caldovic, unpublished). These results suggest that a major contributor to the decrease in CAT gene expression in the developing zygotes was degradation of template, rather than repression of transcription.

Discussion

Our results indicate that expression vectors previously tested in tissue-cultured mouse cells behaved differently during development in the zebrafish and goldfish model systems (Table 1). The major differences in expression of the β-actin/CAT constructs in zebrafish and goldfish were: (1) overall expression was about 100-fold higher in embryos and hatchlings of goldfish than zebrafish; (2) the

Table 1. Expression of transgenic constructs in fish

Construct	*Zebrafish*[a]	*Goldfish*	*Mouse cells*[b]
1	0.4 (100)	215[c] (100)	100
2	2.3 (600)	187[c] (87)	500
3	0.2 (50)	0.5 (0.3)	7[d]
4	0.1 (25)	158 (73)	80[d]

[a]Values in parentheses are normalized to construct 1.
[b]Data taken from Liu *et al.*, 1990c except where noted.
[c]Constructs have the 3'-end of the salmon growth hormone gene replacing the SV40 3'-poly(A) region.
[d]Data taken from Liu *et al.*, 1991 and normalized to Liu *et al.*, 1990c.

first intron had an enhancing effect on gene expression in zebrafish and in some tissue-cultured cell lines but not in goldfish; and (3) the CArG element in the proximal promoter was required for maximal expression of the CAT gene in constructs with just the proximal promoter in both zebrafish and goldfish but, in constructs also containing the first intron, the CArG element was

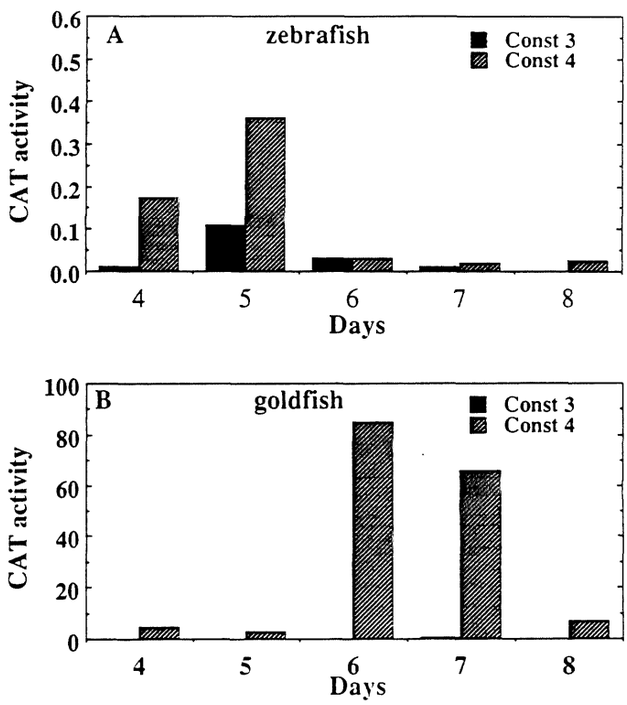


Fig. 4. CAT activity per embryo or hatchling from constructs lacking the CArG box region in the proximal promoter microinjected into zygotes as described in Fig. 2. Construct designations (Fig. 1) are given in the upper right corner of each panel. Expression from constructs 3 and 4 are presented in each panel.

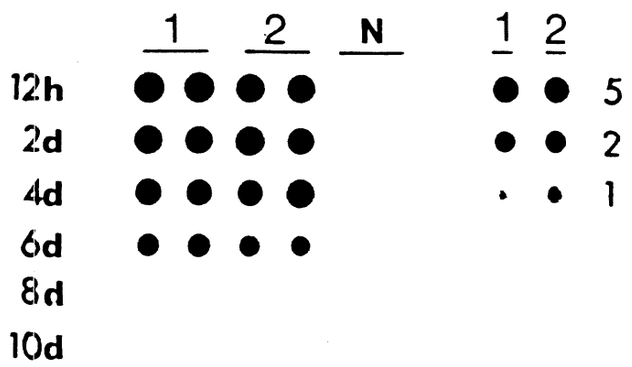


Fig. 5. Persistence of transgenic constructs in zebrafish zygotes. Approximately 5 × 10^5 copies of either constructs 1, 1a, and 2 were microinjected into fertilized eggs as described in Fig. 2. 12 h 20 embryos and at 2, 4, 6, 8 and 10 days post-fertilizat 15 hatchlings were taken and their DNA was extracted, pooled and split into two fractions for duplicate analysis by dot-blotting, using [^{32}P]-labelled CAT gene as a probe. In the left portion of the autoradiogram, duplicate samples of DNA from zygotes injected with either constructs 1 or 2 are shown; N indicates DNA samples taken from uninjected zygotes. In the right third of the figure, dilution series of constructs were included for a blotting standard; the amounts of DNA applied were 1, 2 and 5 × 10^n molecules of each construct.

dispensable for high CAT activity in goldfish but not in zebrafish. This suggests that in the zebrafish there may be interactions between transcriptional elements in the proximal promoter and the first intron (Liu *et al.*, 1991), but not in some cultured rainbow trout cells (Moav *et al.*, 1992b) or goldfish, where a single CArG element sufficed for maximal transcription.

The consistent levels of expression in several cases when different constructs were used and the uniformity of the duration of gene expression indicate the relatively high reproducibility of the experiments. By pooling 10-embryos or hatchlings for each measurement, we ha determined the average activity of the test plasmids following 10–20 separate microinjection into 10–20 individual zygotes. We have not investigated the variation in CAT gene expression between individual microinjected eggs for each of these constructs; however, expression of constructs 1 and 2 has been relatively constant in zebrafish, when normalized per injected zygote, over the course of 10 months when the pool size varied from 4–20 embryos (B. Moav, unpublished).

The single most important finding was the distinct differences in two species of warm water fish in the roles played by the putative transcriptional control elements during early development. We had noted previously variations in transcriptional responses to the intron element in different tissue-cultured cell lines; in mouse L cells and epithelial carp cells (EPC) the intron enhanced

Table 2. Persistence of constructs after microinjection into embryos

Days of development	*Construct (Molecules per embryo × 10^{-5})*	
	1	*2*
0.5	7.5	3.0
2.0	4.6	6.4
4.0	2.2	3.3
6.0	1.0	1.3
8.0	0.2	0.3
10.0	bmd	bmd

bmd: below the minimal level of detection; less than 0.05×10^5 molecules/embryo.
Calculation of the average number of molecules per embryo was done by anning densitometry of the black dots on the x-ray film in Fig. 5, using r calibration known number of molecules of the corresponding constructs, and dividing by the number of embryo equivalents of DNA per dot.

expression 3- to 5-fold, whereas in rainbow trout hepatoma (RTH149) and gonad cells (RTG2) expression was reduced 80% (Moav *et al.*, 1992b). These effects were assumed to be due to differences in available *trans*-acting factors that bind to sequences in the proximal promoter and intron elements (Liu *et al.*, 1991; Moav *et al.*, 1992b). The results presented here suggest that, contrary to our initial expectations, the relative levels of gene expression in equivalent tissues may differ during development in goldfish and zebrafish. The results shown here and those obtained from tissue-cultured cell lines from different species (Moav *et al.*, 1992b) demonstrate that identical transgenic constructs may behave differently in closely related species. There are precedents for common genes to require species-specific transcriptional factors; for example, the rRNA promotors of mammals have minor variations that demand species-specific anscriptional factors (Bell *et al.*, 1990).

The major question that emerges from these experiments concerns the temporal regulation of the β-actin control elements. The expression from each construct should be due to a combination of the number and geometry of *cis*-acting transcriptional control regions and the *trans*-acting transcriptional factors available in the different cells at different times in the developing fish. The differences in duration of activity between the goldfish and zebrafish may be the result of differences in availability of *trans*-acting transcriptional factors and/or differences in the pace of development in the two species. In northern pike and walleye, which develop more slowly at lower temperatures, transgene activity is maintained for more than three weeks (Moav *et al.*, 1992a). Zebrafish were grown at 28° C and hatched in 3–4 days, goldfish were grown at 20-22° C and hatched in 4–5 days, and northern pike and walleye were grown at 11° C and hatched in 9–14 days, respectively. Thus, perhaps not surprisingly, the transgenic DNA appears to have periods of expression that may be related to stage of development in different species of fish.

Our constructs did not contain all of the known control elements for the β-actin gene (Ng *et al.*, 1989; Seiler-Tuyns *et al.*, 1984; Petropoulos *et al.*, 1989). Sequences more than 2.2 kb upstream, which were previously shown to have minor effects on transcription (Liu *et al.*, 1990b), were not examined and none of our constructs contained the conserved motif at the end of the β-actin gene (DePonti-Zilli *et al.*, 1988; Liu *et al.*, 1990a) which is apparently responsible for suppressing β-actin gene transcription during myogenesis. Omission of this negative element should not have reduced CAT gene transcription in our studies.

Our results demonstrate the value of using fish as a model system for investigation of early gene expression. Hundreds of microinjected zygotes, obtained easily and inexpensively, were used for this investigation. This study is the initial investigation into the use of transcriptional control regions from the carp β-actin gene for studying gene expression in developing fish. The next step is the linkage of another reporter gene such as *lac*Z (e.g., Westerfield *et al.*, 1992) to the various vectors to determine tissue specificity and sites of gene expression *in situ* during early growth and development.

Acknowledgements

We thank Aris Moustakas, Jeff Essner, and Scott Fahrenkrug for discussions, and Mark Dalton (University of Minnesota Molecular Biology Computing Center) for help with the figures. This work was sponsored by Public Health Service (NIH) grant CA43881, and grants from the Legislative Committee for Minnesota Resources and the Minnesota Sea Grant College Program (paper JR 272), supported by the National Oceanographic and Atmospheric Administration, Office of Sea Grant, under grant NOAA-86A-D-SG112, project R/A-6. Z.J.L. was supported by a stipend from Minnesota Sea Grant.

References

Bell, S.P., Jantzen, H.-M. and Tjian, R. (1990) Assembly of alternative multiprotein complexes directs rRNA promoter selectivity. *Genes Dev.* **4**, 943–54.

Boxer, L.M., Prywes, R., Roeder, R.G. and Kedes, L. (1989) The sarcomeric actin CArG-binding factor is indistinguishable from the c-fos serum response factor. *Mol. Cell. Biol.* **9**, 515–22.

Chow, K.-L. and Schwartz, R.J. (1990) A combination of closely associated positive and negative *cis*-acting promoter elements regulates transcription of the skeletal α-actin gene. *Mol. Cell. Biol.* **10**, 528–38.

DePonti-Zilli, L., Seiler, Tuyns, A. and Paterson, B.M. (1988) A 40-base-pair sequence in the 3′ end of the β-actin gene regulates β-actin mRNA transcription during myogenesis. *Proc. Natl Acad. Sci. USA* **85**, 1389–93.

Du, S.J., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R. and Hew, C.L. (1992) Growth enhancement in transgenic Atlantic salmon by the use of an "all-fish" chimeric growth hormone gene construct. *Bio/Technology* **10**, 176–81.

Fletcher, G.L. and Davies, P.L. (1991) Transgenic fish for aquaculture. *Genetic Engineering* **13**, 331–70.

Friedenreich, H. and Schartl, M. (1990) Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. *Nucl. Acids Res.* **18**, 3299–305.

Gong, Z., Hew, C.H. and Vielkind, J.R. (1991) Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese medaka embryos. *Mol. Mar. Biol. Biotech.* **1**, 64–72.

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**, 1044–51.

Hackett, P.B. (1992) The molecular biology of transgenic fish. In Hochachka, P. and Mommsen, T., eds, *The Biochemistry and Molecular Biology of Fishes.* Volume 2 (in press).

Hew, C.L. (1989) Transgenic fish: present status and future directions. *Fish Physiol. Biochem.* **7**, 409–13.

Hew, C.L., Trinh, K.Y., Du, S.J. and Song, S. (1989) Molecular cloning and expression of salmon pituitary hormones, *Fish Physiol. Biochem.* **7**, 375–80.

Holtfreter, J. (1931) Uber die isolierter teile des amphibienkeimes. II. Zuchtung von keimen und keimteilen in salzlosung. *Wilhelm Roux' Archiv fur entwicklungsmechanik der organismen.* **124**, 404–66.

Kawamoto, T., Makino, K., Niwa, H., Sugiyama, H., Kimura, S., Amenmura, M., Nakata, A. and Kakunaga, T. (1988) Identification of human β-actin enhancer and its binding factor. *Mol. Cell. Biol.* **8**, 267–72.

Kimmel, C.B. (1989) Genetics and early development of zebrafish. *Trends Genet.* **5**, 283–8.

Liu, Z., Zhu, Z., Roberg, K., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1990a) The isolation and characterization of the β-actin gene of carp (*Carpinus carpio*). *DNA Sequence* **1**, 125–36.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1990b) Functional analysis of elements affecting expression of the β-actin gene of carp. *Mol. Cell. Biol.* **10**, 3432–40.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1990c) Development of vectors for transgene expression in fish. *Bio/Technology* **8**, 1268–72.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1991) Importance of the *CArG* box in regulation of β-actin-encoding gene. *Gene* **108**, 211–17.

Minty, A. and Kedes, L. (1986) Upstream regions of the human cardiac actin gene that modulate its transcription in mouse cells: presence of an evolutionarily conserved repeated motif. *Mol. Cell. Biol.* **6**, 2125–36.

Mitchell, P.J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371–8.

Moav, B., Liu, Z., Moav, N., Gross, M.L., Kapuscinski, A.R., Faras, A.J., Guise, K.S. and Hackett, P.B. (1992a) Expression of heterologous genes in transgenic fish. Hew, C. and G.L. Fletcher, eds, In *Transgenic Fish* pp. 121–140. World Scientific Pub. Co.

Moav, B., Liu, Z., Groll, Y. and Hackett, P.B. (1992b) Selection of promoters of transgenic fish. *Mol. Mar. Biol. Biotech.* **1**, 338–45.

Ng, S.-Y., Gunning, P., Liu, S.-H. Leavitt, J. and Kedes, L. (1989) Regulation of the human β-actin promoter by upstream and intron domains. *Nucl. Acids Res.* **17**, 601–15.

Orita, S., Makino, K., Kawamoto, T., Niwa, H., Sugiyama, H. and Kakunaga, T. (1989) Identification of a site that mediates transcriptional response of the human β-actin gene to serum factors. *Gene* **7**, 13–9.

Petropoulos, C.J., Rosenberg, M.P., Jenkins, N.A., Copeland N.G. and Hughes, S.H. (1989) The chicken skeletal mus α-actin promoter is tissue specific in transgenic mice. *M. Cell. Biol.* **9**, 3785–92.

Pinkert, C.A., Ornitz, D.H., Brinster, R.L. and Palmiter, R.D. (1987) An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. *Genes Dev.* **1**, 268–76.

Powers, D. (1989) Fish as model systems. *Science* **246**, 352–8.

Ptashne, M. (1988) How eukaryotic transcriptional activators work. *Nature* **335**, 683–9.

Rossant, J. and Hopkins, N. (1992) Of fin and fur: mutational analysis of vertebrate embryonic development. *Genes Dev.* **6**: 1–13.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor NY: Cold Spring Harbor, Laboratory Press.

Schartl, M., Wittbrodt, J., Maueler, W., Raulf, F., Adam, D., Hannig, G., Telling, A., Storch, F., Andexinger, S. and Robertson, S.M. (1990) In Schroder, J.H. and Schartl, M., eds, *New Trends in Icthyology*, Parey Verlag (in press).

Schwab, M. (1987) Oncogenes and tumor suppressor genes in *Xiphophorus. Trends Genet.* **3**, 38–42.

Seiler-Tuyns, A., Eldrige, J.D. and Paterson, B.M. (198[illegible] Expression and regulation of chicken actin genes introdu into mouse myogenic and nonmyogenic cells. *Proc. Natl Acad. Sci. USA* **81**, 2980–4.

Shears, M.A., Fletcher, G.L., Hew, C.L., Gauthier, S. and Davies, P.L. (1991) Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). *Mol. Mar. Biol. Biotech.* **1**, 58–63.

Subramaniam, M., Schmidt, L.J., Crutchfield, C.E. and Getz, M.J. (1989) Negative regulation of serum-responsive enhancer elements. *Nature* **340**, 64–6.

Swift, G.H., Kruse, F., MacDonald, R.J. and Hammer, R.E. (1989) Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. *Genes Dev.* **3**, 687–96.

Tronche, F., Rollier, A., Bach, I., Weiss, M.C. and Yaniv, M. (1989) The rat albumin promoter: cooperation with upstream elements is required when binding of AFP/HNF1 to the proximal element is partially impaired by mutation or bacterial methylation. *Mol. Cell. Biol.* **9**, 4759–66.

Walsh, K. (1989) Cross-binding of factors to functionally different promoter elements of c-*fos* and skeletal actin genes. *Mol. Cell. Biol.* **9**, 2191–201.

Westerfield, M. (1989) *The Zebrafish Book*, Eugene: University of Oregon Press.

Westerfield, M., Wegner, J., Jegalian, B.G., DeRobertis, E.M. and Psuchel, A.W. (1992) Specific activation of mammalian *Hox* promoters in mosaic zebrafish. *Genes Dev.* **6**, 591–8.

Winkler, C., Vielkind, J.R. and Schartl, M. (1991) Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). *Mol. Gen. Genet.* **226**, 129–40.

Xiong, F., Chin, R.A. and Hew, C.L. A gene encoding chinook salmon (*Oncorhynchus tschawytscha*) prolactin: gene structure and potential *cis*-acting regulatory elements. (1992) *Mol. Mar. Biol. Biotech.* **1**, 155–164.

Molecular Marine Biology and Biotechnology (1993) 3(2) 162–173

Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein

Zoltán Ivics,* Zsuzsanna Izsvák,† and Perry B. Hackett‡

Department of Genetics & Cell Biology, University of Minnesota, St. Paul, MN 55108, U.S.A.

Abstract

Manufacture of lines of fish containing specific transgenes is difficult because most fish that hatch from embryos injected with foreign DNA are mosaic; few have the transgenic DNA integrated in germ-line cells. To determine whether the process of integration of exogenously supplied DNA into fish genomes could be accelerated, we examined the ability of the Moloney murine leukemia virus (MoMLV) integration protein (IN) to function in embryonic zebrafish cells. We used partially purified IN from a baculovirus/insect cell expression system and unpurified IN from extracts of ψ-2 mouse cells that carry a MoMLV provirus. Both forms of IN were able to enhance expression in zebrafish 10 days after fertilization. At day 14 of development, fish injected with IN had higher levels of transgenic DNA than control fish. The ability of IN to enhance integration of transgenic constructs was demonstrated by a ligation-medhyated polymerase chain reaction procedure, which was employed to detect junction fragments of foreign and host genomic DNA, generated by IN-mediated integration.

Introduction

In the past few years, great efforts have been made to develop methods of producing transgenic fish that have incorporated specific genes into their chromosomes (Fletcher and Davies, 1991). However, inefficient and delayed integration of exogenously supplied DNA into fish chromosomes has impeded establishment of lines of transgenic fish (Hackett, 1993). Most fish that hatch from embryos microinjected with extra genes are mosaics with respect to the chromosomal locations and copy numbers of integrated transgenes (Stuart et al., 1988; Culp et al., 1991). When the transgenic DNA does not integrate into the chromosomes of germline cells, transmittance of the transgenic genotype is rare and unstable. Screening for those progeny that carry transgenic DNA is tedious and inefficient. To address these problems associated with delayed integration, we initiated development of methods for accelerating the rate of early integration of transgenic DNA into fish genomes.

Our previous work with retroviruses alerted us to the efficiency and other attractive features of retroviral integrase proteins for insertion of extrachromosomal DNA into chromosomes. Soon after entry into the host cell, a complementary DNA (cDNA) copy of the retroviral RNA genome is integrated into host chromosomes to form a provirus (Varmus and Brown, 1989; Brown, 1990; Grandgenett and Mumm, 1990). The integration process is mediated by a virus-encoded integrase (IN) protein. Retroviral integrases preferentially insert cDNA into transcriptionally active chromosomal regions (Shih et al., 1988), in contrast to random recombination, which can occur with transcriptionally silent chromatin (Sato, 1992), and they do not show any obvious target-site specificity (Craigie, 1992). Although nucleosomal DNA is not accessible to many DNA-binding proteins (Gross and Garrard, 1988; Grunstein, 1990), integrases preferentially target nucleosomal structures over naked DNA (Pryciak et al., 1992a, 1992b; Pryciak and Varmus, 1992). General recombination of transgenic DNA into chromosomes can lead to rearrangements of the inserted sequence, which may destroy expression of the gene (Gridley et al., 1987). Integration by retroviral IN proteins should avoid this problem because the inserted DNA must

*Correspondence should be sent to this author.

†Permanent addresses: Institute of Biochemistry, Biological Research Center of Hungarian Academy of Sciences, Szeged, Hungary; and ‡Institute of Human Genetics, University of Minnesota, Minneapolis, MN 55455, U.S.A.

be intact with flanking IN recognition sequences (INRSs). IN accomplishes the integration reaction in three steps: (1) recognition of the INRSs and removal of two nucleotides from the 3′ ends; (2) cleavage of the chromosomal DNA; and (3) joining of the viral cDNA into the host chromosome (Craigie et al., 1990). The integration reaction could be assayed in cell-free systems (Craigie et al., 1990; Katz et al., 1990; Bushman and Craigie, 1991; Engelman et al., 1991), and the INRS specificities of a number of integrases have been determined (Sherman et al., 1992; Bushman and Craigie, 1990), including that of the Moloney murine leukemia virus (MoMLV) IN (Bushman and Craigie, 1990), which we chose to investigate for this project.

The prospective use of IN to accelerate integration raised two major questions. First, can an integrase from a nonpiscine source work in fish? A number of different recombinase systems have been used in organisms other than their natural host with sporadic success. Successful work has been done with several recombinases from plants in heterologous plant species (Haring et al., 1991), wth the yeast FLP recombinase in *Drosophila* and mammalian cells (Golic and Lindquist, 1989; O'Gorman et al., 1991), and with the Cre recombinase of bacteriophage P1 in transgenic mice (Orban et al., 1992). However, not all such attempts have brought success, perhaps due to the inactivity of the recombinase (Rio et al., 1988; Kaufman and Rio, 1991). In all these experiments, a gene encoding a recombinase protein was transferred into cells. Expression of the recombinase then permitted mobilization of DNA having the necessary *cis*-acting elements for recognition by the enzyme.

The second question was should either a gene encoding an integrase (recombinase) or just the protein itself be injected with the substrate DNA that we wanted integrated into fish genomes? Kaufman and Rio (1991) first reported the transfer of protein, P-transposase, plus a substrate DNA for recombination. However, this experiment was done in *Drosophila*, the natural system for P-transposase. Use of IN protein instead of its gene was essential in our experiments because transcription of a gene encoding IN would presumably not occur before the midblastula transition stage (Kimmel, 1989), when the developing fish embryo consists of more than 1,000 cells. This would result in a delayed production of integrase and presumably cause a greater degree of mosaicism. In our experiments, we used IN protein that was previously tested for activity, thereby improving the chance that it would be active immediately after microinjection. An additional advantage of using IN protein is that its catalytic lifetime is relatively short because in natural systems its presence is deleterious to genomic stability. These results suggested that a heterologous recombinase protein could be functional in developing fish embryos.

Accordingly, using zebrafish (*Brachidanio rerio*) as a model system, we examined the potential of the IN protein from MoMLV to accelerate the rate of integration of transgenic DNA into fish chromosomes. We transferred mixtures of IN and reporter gene constructs into early zebrafish embryos and found that the viral protein was capable of elevating both integration and expression of exogenously supplied DNA.

Results

MoMLV IN activity in vitro

Both IN and appropriate substrate DNAs for integration were required for this project. The IN used in our experiments was obtained from two sources: BV/IN from a baculovirus-insect cell expression system; and ψ-2/IN from extracts of ψ-2 mouse cells, which carry an integrated, replication-defective MoMLV provirus (Mann et al., 1983). Approximately 50 mg BV/IN were partially purified from the baculovirus-infected cells as described by Craigie and associates (1990), with some modifications to increase stability and activity (Izsvak et al., unpublished observations). Because the ψ-2 cell line produces all of the retroviral proteins required for virus propagation (Mann et al., 1983), we assumed that it would produce functional IN. Total protein extract from ψ-2 cells was prepared without further purification of IN. Construction of suitable reporter substrates was necessary to assay the integration activities of BV/IN and ψ-2 integrase. To mimic unintegrated retroviral DNA, the substrate DNAs had to be linear, with specific INRSs at both termini that are necessary for integration (Bushman and Craigie, 1990). We used the wild-type MoMLV INRS with a single base change that introduced an *Nde*I restriction endonuclease site at the end of the INRS (Fujiwara and Craigie, 1989) (Figure 1). This single base mutation in the INRS is tolerated during virus replication *in vivo* (Colicelli and Goff, 1988). Any reporter gene with an appropriate promoter could be introduced between the two INRSs to become a substrate for MoMLV IN (Fujiwara and Craigie, 1989). Thus, cleavage of substrate plasmids

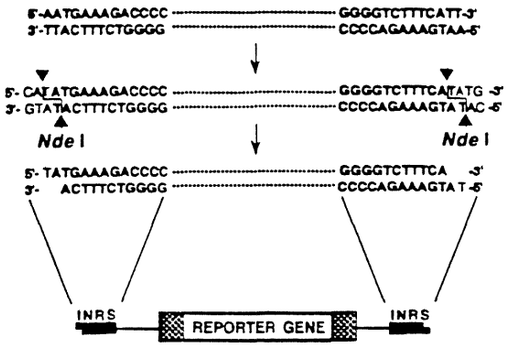
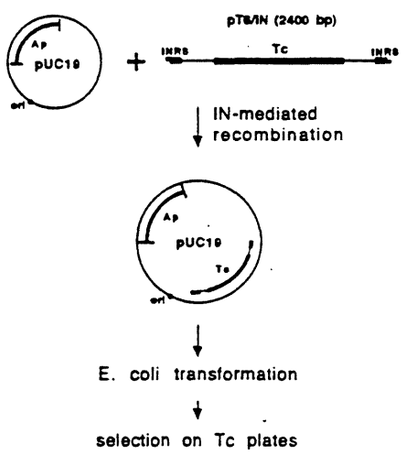


Figure 1. General structure of a stubstrate for MoMLV IN. Substrates were linear fragments with integrase recognition sequences (INRS) at both ends. The top fragment shows the structure of a wild-type cDNA substrate for the MoMLV IN. The wild type INRSs were mutated to incorporate *Nde*I recognition sites for linearization and release from parental plasmids. The IN substrates also carried different reporter genes, whose expression could conveniently be assayed. The single base mutation and the recessed 3'-ends are shown.

with *Nde*I restriction endonuclease permitted us to recover them in a linear form, with appropriate terminal INRSs with 3' recessed ends, similar to the immediate precursor for natural viral integration (Craigie et al., 1990) (See Figure 1).

Before examining the effect of integrase on transgene integration in fish, we had to determine the integration activities of the two IN preparations *in vitro*. Our assay for IN activity, using the pT6/IN construct containing the tetracycline resistance gene (Tc) (Figure 2A), was based on a genetic approach similar to that developed by Fujiwara and Craigie (1989). The assays showed the ability of IN to mediate integration of the pT6/IN substrate DNA into plasmid pUC19, thereby producing a recombinant molecule that could be detected on selective plates following transformation of bacteria with the products of the integration assay. The molar ratio of substrate DNA to BV/IN in these experiments was 1:30 to 50, similar to Bushman and Craigie (1990). Figure 2B shows that integration activity of BV/IN yielded 16 detectable recombinant plasmids in 10^6 target molecules that were screened. Because the

a.



b.

Sample	Number of integrants per 10^6 target molecules
DNA + LMTK⁻ extract	0
DNA + BV/IN + LMTK⁻ extract	16
DNA + ψ-2 extract	7

Figure 2. Integration efficiency of IN *in vitro*. (A) pUC19 served as a target and a 2,400-bp fragment of pT6/IN, carrying the tetracycline resistance gene and terminal INRSs, was the substrate for IN in the *in vitro* integration assay. Reaction products were transformed into *E. coli*, and the recombinants were identified on agar plates containing tetracycline. (B) *In vitro* integration efficiency of BV/IN and ψ-2/IN. Either 5 μL from the BV/IN preparation or 40 μL ψ-2 cell extract were used as sources of integration activity. An extract of LMTK⁻ mouse cells was used as a control. Data are from three independent experiments.

ratio of substrate pT6/IN to target pUC19 molecules was 1:2, 16 of 5×10^5 pT6/IN molecules were able to integrate. Although seemingly low, this rate of integration corresponds to frequencies reported by others using MoMLV IN *in vitro* (Brown et al., 1987; Fujiwara and Craigie, 1989). The integration activity of ψ-2/IN *in vitro* has not been previously reported. We were able to detect integration activity from the ψ-2 cell extract at approximately the same level as from the BV/IN under the conditions we used (see Figure 2b). Recombinant clones resistant to tetracycline were confirmed by restriction analysis (not shown). A control extract of mouse LMTK$^-$ cells, the parental line of ψ-2 cells, *produced no recombinant plasmids (see Figure 2b), indicating that the recombination events we recorded were specific for BV/IN and the* ψ-2 cell extract.

These results allowed us to estimate the level of active IN in the 2 protein preparations. Following purification, the BV/IN concentration was estimated to be approximately 1 pmol/μL, whereas the IN concentration in the ψ-2 cell extract was less than 5×10^{-4} pmol/μL, the threshold for detection on a silver-stained protein gel (data not shown). Because the activities of the 2 preparations were nearly the same, we conclude that the concentration of active BV/IN molecules was less than 1%. The poor specific activity of BV/IN activity can be due in part to inefficient renaturation of IN following its isolation from the inclusion bodies in the insect cells.

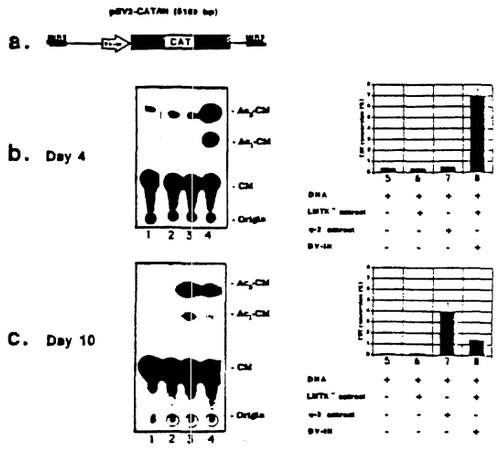


Figure 3. Effect of MoMLV integrase on expression of transgenic DNA in zebrafish. Zebrafish embryos at the 1- to 2-cell stage injected with *Nde*I-linearized pSV2-CAT/IN construct (A) either with or without IN. CAT activity was assayed at days 4 (B) and 10 (C). Total protein extracts from 50 to 60 fish were pooled, and aliquots equivalent to approximately 15 fish were used for each assay. Samples are as follows: lanes 1 and 5, pSV2-CAT/IN; lanes 2 and 6, pSV2-CAT/IN + LMTK$^-$ extract; lanes 3 and 7, pSV2-CAT/IN + ψ-2 extract; lanes 4 and 8, pSV2-CAT/IN + LMTK$^-$ extract + BV/IN. Data are from three independent experiments; bars show the standard errors for each condition.

MoMLV IN activity in zebrafish embryos

Having established the activities of the 2 sources of IN *in vitro*, our next step was to examine IN activity in fish. Approximately 2,000 zebrafish embryos at the 1 to 2 cell stage were injected with a mixture of IN and DNA substrates. The molar ratio of substrate DNA to BV/IN in these injections was the same as that used in the *in vitro* experiments. The amount of ψ-2 cell extract used for microinjection was adjusted to provide approximately the same integration activity as the BV/IN preparation.

Our first experiment in fish was designed to measure any changes in gene expression due to the presence of IN in the coinjections of protein and DNA. The substrate DNA, pSV2-CAT/IN (Figure 3A), used in these experiments had the bacterial chloramphenicol acetyltransferase (CAT) reporter gene behind an SV40 enhancer/promoter element in place of the Tc gene used in the *in vitro* assays. The *Nde*I-linearized pSV2-CAT/IN reporter construct was transferred either alone or together with LMTK$^-$ cell extract in control treatments, and together either with ψ-2 cell extract or a mixture of LMTK$^-$ cell extract plus BV/IN. The LMTK$^-$ cell extract was included as a supplement to BV/IN because mouse cellular proteins have been shown to have a stimulatory effect on integration efficiency (Fujiwara and Craigie, 1989). Protein extracts were prepared from pools of 50 to 60 fish taken at 4 and 10 days after fertilization/microinjection (see Figure 3). We chose these days for sampling because at day 4, transient expression is usually high, whereas by day 10, most of the extrachromosomal DNA is lost in the developing animal, and transient expression measured at this time is generally weak or undetectable (Chong and Vielkind, 1989; Winkler et al., 1991; Moav et al., 1993).

At day 4, CAT expression could be measured in all of the treatments (see Figure 3B), as expected from microinjected DNA with a strong constitutive promoter (Liu et al., 1990; Winkler et al., 1991;

Moav et al., 1993). However, CAT activity was 15-fold higher in embryos injected with BV/IN (see Figure 3B, lanes 4 and 8), in contrast to those injected with DNA only or DNA together with LMTK$^-$ or ψ-2 cell extracts (see Figure 3B, lanes 1–3 and 5–7). Because the ψ-2 extract did not seem to have any detectable influence on transient CAT expression, we concluded that the effect caused by the BV/IN was not due to a specific integrase action but rather to some property of the BV/IN preparation (see Discussion). Analysis of transgenic DNA at day 4 by dot blotting showed that the elevated level of transient CAT expression by the BV/IN preparation was probably the result of increased persistence of transgenic DNA in fish coinjected with DNA and BV/IN (data not shown).

Analysis of CAT activities from samples taken at day 10 revealed a specific effect of integrase on transgene expression (see Figure 3C). CAT expression was 40-fold higher in samples injected with ψ-2 cell extract and 14-fold higher with BV/IN (see Figure 3c, lanes 3, 4, and 7, 8) than in control embryos injected with either DNA or DNA plus LMTK$^-$ cell extract (see Figure 3c, lanes 1, 2, and 5, 6). In a parallel study using a construct without flanking INRS elements, there was no increase in expression at day 10 when integrase was coinjected (data not shown). We conclude that the differences observed in CAT expression were the consequence of IN because LMTK$^-$ cell extract alone did not have any effect at either time. In contrast to the results from day 4, at day 10, both IN sources were effective in increasing CAT expression. We presume that the IN-specific enhancement of expression observed at day 10 may have been masked at day 4 by the relatively high expression of unintegrated reporter constructs that greatly exceeded the number of integrated copies of the substrate DNA.

The experiment suggested that more copies of microinjected DNA were being stabilized in chromatin as a result of IN activity. A consequence of an increased uptake of exogenous DNA into chromosomes would be an increase in integrated DNA several weeks after microinjection, due to its replication during cell division in the growing fish. At this time, the cellular copy number of unintegrated DNA is reduced by more than 99% (Moav et al., 1993). To determine whether IN increased the level of transgenic DNA in zebrafish 2 weeks after microinjection, we used another INRS-containing construct, pRSVTk-Neo/IN, that had a neomycin phosphotransferase (*neo*) gene behind a Rous sarcoma virus enhancer/herpes simplex virus thymidine kinase promoter. This construct was chosen because in the future it might allow us to assay further integration events by genetic selection of fish that incorporated the transgenic *neo* gene into the chromosomes of every cell, which as yet has not been accomplished (Yoon et al., 1990).

The *Nde*I-linearized pRSVTk-Neo/IN construct (see Figure 4A) was injected into the cytoplasms of early zebrafish embryos at the 1 to 2 cell stage either (1) with LMTK$^-$ cell extract in control experiments, (2) together with ψ-2/IN, or (3) with a mixture of BV/IN and LMTK$^-$ cell extract. DNA samples were prepared from fish killed at 14 days, an age when extrachromosomal DNA cannot be detected by DNA hybridization (Fletcher and Davies, 1991). The amounts of transgenic DNA in 125 fish from each of the 3 treatments were measured by dot blotting. To determine the amounts of pRSVTk-Neo/IN in each group of microinjected embryos we performed the following steps. First, the hybridization signals from DNA obtained from nonmicroinjected fish were determined and the highest value was designated as background. Then each DNA sample from the three groups of microinjected embryos was evaluated relative to background. The results are shown in Figure 5, where the number of fish with detectable amounts of transgenic DNA are displayed as a function of the hybridization signal intensity of their DNA above background, as determined by densitometric scanning. Two features of the data in Figure 5 are consistent with enhanced integration of exogenous DNA by IN. First, the levels of transgenic DNA in fish hatched from embryos injected with either ψ-2/IN or BV/IN were significantly higher than that from the LMTK$^-$ control group without IN (α=0.05 by the *t*-test). Twenty-two (17% of the 125 fish sampled) control samples from fish injected with DNA plus LMTK$^-$ cell extract yielded hybridization signals above background (see Figure 5A). In contrast, 40 fish (32%) of the ψ-2/IN treatment group (see Figure 5B) and 53 fish (42%) of the BV/IN treatment group (See Figure 5C) had hybridization signals above background. Second, stronger signals were obtained from fish into which DNA plus IN were coinjected (see Figure 5B, C), suggesting that the transgenic fish had either a higher copy number or more cells with transgenic DNA. This analysis of relatively large numbers of samples by DNA do blotting and hybridization provided good statistical evidence that MoMLV IN enhanced transgene persistence.

However, although dot blotting served well as a

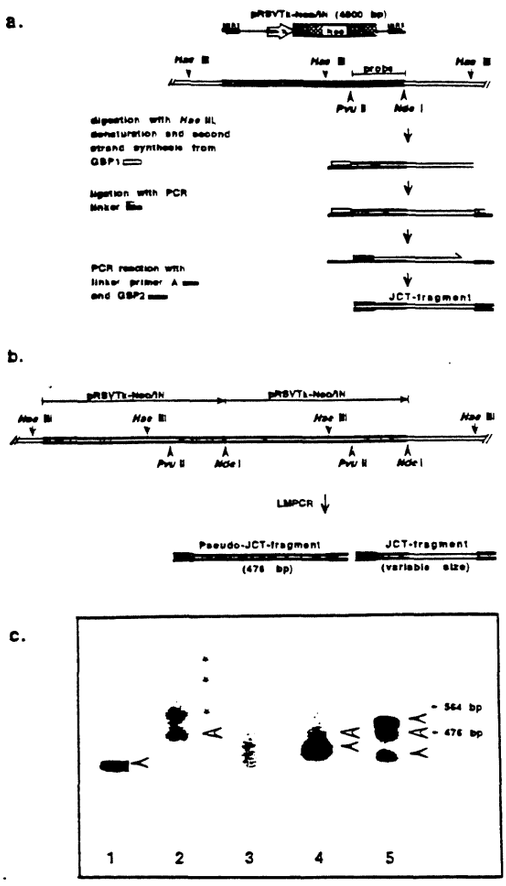


Figure 4. LMPCR detection of chromosomal integration events. (A) Amplification of a junction (JCT) fragment formed by a single copy of integrated transgenic pRSVTk-Neo/IN and flanking chromosomal sequences. Genomic DNA from 14-day-old fish was digested completely by *Hae*III restriction endonuclease. Gene-specific primer 1 (GSP1) was annealed to denatured DNA, and second strand synthesis produced blunt-ended molecules. PCR linkers were ligated to the blunt ends, and the DNA sequences were amplified using Linker Primer A and Gene-specific Primer 2 (GSP2), whose binding site was internal to that of GSP1. Amplification products were Southern blotted and hybridized (not shown) with a 290-bp *Pvu*II-*Nde*I fragment of pRSVTk-Neo/IN. (B) Amplification of a pseudojunction and a junction fragment resulting from integration of tandemly repeated transgene copies. In this instance, results from an integrated dimer are illustrated; a 476-bp fragment and a variable length fragment would be produced. (C) PCR reactions of DNA from selected fish (see Figure 4) were electrophoresed on a 0.9% agarose gel, blotted, and hybridized with the 290-bp *Pvu*II-*Nde*I probe. Filled arrowheads represent chromosomal junction fragments, indicative of integration events; *empty arrowheads* mark the 476-bp pseudojunction fragment resulting from transgenic multimers; and *asterisks* indicate background bands that appeared in all lanes after long exposure. Samples are as follows: lane 1, control from a stable transgenic mouse cell line; lane 2, control from fish injected with DNA + LMTK$^-$ extract; and lanes 3–5, DNA from three fish injected with DNA + LMTK$^-$ extract + BV/IN. Exposure times were as follows: lanes 1, 3, 4, and 5, 6 hours; lane 2, 2 days. The positions of a 564-bp lambda DNA *Hin*dIII fragment and the 476-bp pseudojunction fragment are indicated in the right margin.

first screen, it did not provide direct evidence for integration of transgenic DNA. For that purpose, we utilized the following strategy. From each of the 3 treatment groups shown in Figure 5, we analyzed 6 samples that had strong hybridization signals (designated by asterisks in Figure 5) by ligation-mediated polymerase chain reaction (LMPCR) (Mueller and Wold, 1989). This method allows efficient detection and molecular cloning of sequences flanking integration sites to document integration events (Izsvak et unpublished observations) (see Figure 4A). Multimers of transgenic constructs, mostly head-to-tail concatemers, are formed in fish cells within minutes of microinjection (Chong and Vielkind, 1989). These multimers can persist for several months in an extrachromosomal state (Stuart et al., 1990; Winkler et al., 1991) at a concentration below detection by the dot-blotting assay shown in Figure 5. However, junctions between transgenes within a multimer can be detected by LMPCR as "pseudojunction fragments" of predictable size (see Figure 4B) so they need not interfere with the identification of true integration events. Although formation of head-to-head and tail-to-tail multimers of transgenic DNA has been shown in fish embryos, their concentration is less than 10% that of tandemly repeated messages (Chong and Vielkind, 1989). Detection of tail-to-tail junctions is not possible due to the positions of PCR primers used in our assay and is problematic in the case of inverted repeats in the head-to-head orientation (Does et al., 1991). Amplification of the terminal region of unintegrated, linear monomers could contribute to the background of the method, but the size of this product is also predictable, thus allowing its identification. The detection of one junction fragment is indicative of a single insertion (see Figure 4A),

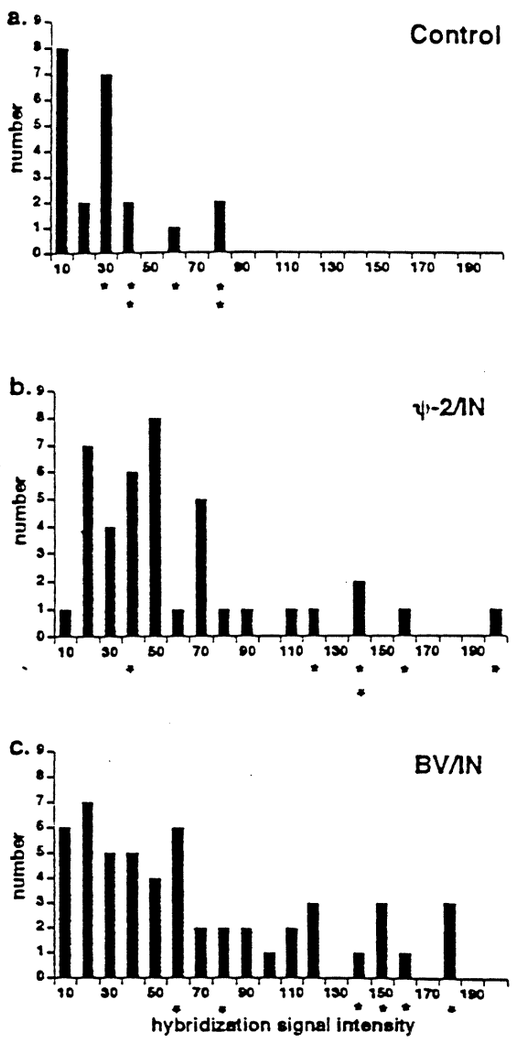


Figure 5. Effect of MoMLV integrase on transgene persistance. Zebrafish embryos were injected with either (A) *Nde*I-linearized pRSVTk-Neo/IN + LMTK$^-$ extract; (B) *Nde*I-linearized pRSVTk-Neo/IN + ψ-2 extract; or (C) *Nde*I-linearized pRSVTk-Neo/IN + LMTK$^-$ extract + BV/IN. DNA samples from 125 fish from each group were isolated and analyzed by dot-blotting. The hybridization background was defined as the maximal signal obtained from uninjected fish DNA and was subtracted from the values of experimental samples. Only signals above background are shown in the histograms. The abscissa indicates the percentage above background of the hybridization signal intensity, and the ordinate indicates the number of fish that had signals at each intensity. *Asterisks* indicate groups from which individual samples for PCR analysis were selected.

whereas presence of more than one junction fragment may represent multiple integrations. Although out of the range of quantitative PCR, the strength of the hybridization signal is suggestive of the copy number of the transgene. Thus, LMPCR can suggest approximately when in embryonic development a genomic integration occurred: delayed integration generally results in a low copy number, whereas early integration is expected to generate a higher copy number of the transgenic DNA.

Accordingly, LMPCR amplification products from the DNAs of the selected fish from the experiment shown in Figure 5 and a positive control sample of DNA from a transgenic mouse cell line were blotted and probed with a 290-bp *Pvu*II-*Nde*I fragment of pRSVTk-Neo/IN. None of the 18 fish analyzed contained unintegrated transgenic monomers. However, most DNA samples revealed the 476-bp pseudojunction fragment that would result from early multimerization of the transgene (see Figure 4C, lanes 2, 4, and 5). Some background bands appeared in all of the samples (designated by asterisks in Figure 5C, lane 2) but only after long exposure. Three samples, one from the ψ-2/IN treatment and 2 from the BV/IN treatment generated smears on the radiogram, which could be the result of multiple insertions that generated a heterogeneous population of chromosomal junction fragments (see Figure 4C, lane 3). In sum, 13 of 18 samples showed putative chromosomal junction fragments, indicating genomic integration events. Eleven of these animals appeared to be mosaics, because the junction fragments appeared as faint bands on blots, which indicates delayed integration.

Two fish that hatched from embryos injected with BV/IN produced exceptionally strong signals. One (see Figure 4C, lane 4) showed a single, prominent band, suggesting a high copy number of the transgene. This finding is consistent with an early integration event into a single chromosomal locus without previous multimerization. The presence of a weak pseudojunction fragment probably reflects the existence of extrachromosomal, low copy number transgene multimers in this animal. The second fish (see Figure 4C, lane 5) could be characterized as having 2 independent integration events plus a strong pseudojunction fragment, suggestive of an integrated multimer. From their intensities, both integration events could have occurred relatively early in development. Because the signal intensities of the pseudojunction frag-

ment and one of the junction fragments seem to be the same, these 2 bands may represent integration of a transgenic dimer.

These results can be regarded as evidence for early integration of microinjected DNA into fish chromosomes. Overall, 2 of the 12 fish surveyed that hatched from embryos injected with IN appeared to have chromosomes with transgenic DNA that had the characteristics expected from IN-mediated integration early in embryogenesis. Results from the expression experiments, dot-blot analyses, and LMPCR studies suggest that MoMLV IN enhances early integration of transgenic DNA into fish genomes.

Discussion

The MoMLV integrase enhances transgene integration and expression in zebrafish

We have shown that the MoMLV integration protein from 2 different sources, a baculovirus-insect cell expression system and the retrovirus packaging cell line ψ-2, enhanced expression and integration in zebrafish following their coinjection with suitable reporter substrates into early embryos. Two experimental approaches suggest that the MoMLV IN enhanced integration into zebrafish DNA. First, analysis of CAT expression 10 days after fertilization, when expression of unintegrated DNA is reduced, revealed that both ψ-2/IN and BV/IN significantly increased the level of expression of transgenic DNA. This appears to be the consequence of IN-mediated integration, because transient background expression was almost undetectable at this time. Second, DNA dot-blot analysis of samples taken from 375 fish at day 14 of development showed that fish injected with IN had higher levels of transgenic DNA than control fish. This finding could represent extended survival of extrachromosomal DNA or more frequent and earlier integration events. We consider it unlikely that increased persistence of extrachromosomal DNA by IN accounts for the large percentage of fish that had concentrations of exogenous DNA above background in the experiment reported in Figure 5, because the results with BV/IN and ψ-2/IN were nearly equal, even though the concentrations of IN in the two injections differed approximately by 1,000-fold. The data are most consistent with IN facilitating integration of transgenic DNA into zebrafish chromosomes.

To test our conclusions further, we used LMPCR to detect junction fragments between transgenic and host genomic DNA. We found integrants that appear to have been generated by IN-mediated integration. In 2 cases, integration may have occurred early in development. One is probably the result of a single transgene and the other may represent 2 independent insertions, including integration of a transgenic dimer, suggesting that multimers can be used as IN substrates because they are linear and have INRS elements. An additional 3 fish were found consistent with multiple integrations in the IN treatment groups. Final proof of IN-mediated chromosomal integration would come from cloning and sequencing the PCR-amplified junction fragments, the identification of target-site duplications flanking the inserted transgenic construct, and Mendelian passage of the transgenes through several generations. Such labor-intensive investigations are underway.

BV/IN enhanced the transient expression from the reporter construct in a nonspecific manner 4 days after fertilization (see Figure 3B). Quite possibly, the large quantity of BV/IN protein was responsible for the effect. Because the concentration of ψ-2/IN was at least 1,000-fold less, we would not have expected to detect such stabilization with this source of IN. IN molecules in excess might protect extrachromosomal transgene copies from host nucleases in a substrate-independent manner, similar to protection of DNA by phage coat in cell transfection experiments (Ishiura et al., 1982; Vielkind and Vogel, 1989). Nonspecific DNA binding affinity of integrase (Roth et al., 1988) and prolonged persistence of transgenic DNA at day 4 in fish coinjected with DNA and BV/IN support this hypothesis. Because the physical protection of the DNA by the protein could elevate the level of transient expression, it may be beneficial to transfer DNA along with an excess of DNA-binding proteins, such as IN, into fish embryos.

Because the IN protein is expected to have only transient activity in fish cells, the period during which IN efficiently functions is limited, as suggested by the results in Figure 3, which show stabilization of DNA at day 4 but not at day 10 after microinjection. Pryciak and colleagues (1992b) noted that cells containing preintegration complexes must undergo a division before integration can take place. Because the exogenously supplied IN was delivered at the 1 to 2 cell stage of development, which is followed by a period of extremely rapid cell division, IN-mediated integration may have a good chance of occurring before degradation of the protein.

Future prospects

Exploiting recombinase proteins from various sources in heterologous eukaryotic systems for insertional mutagenesis, transposon-tagging, and targeted gene replacement is under investigation on a number of fronts (Rossant and Hopkins, 1992). In plants, heterologous transposon systems have been successfully employed (Haring et al., 1991). In animal cells, only site-specific recombinases from prokaryotes and yeast have been reported to accomplish complete transposition (Golic and Lindquist, 1989; O'Gorman et al., 1991; Orban et al., 1992). These recombination systems can be excellent tools for targeted chromosomal insertions, but their target-site specificity narrows the range of their applicability for random insertional mutagenesis. The application of recombinase proteins in heterologous organisms, although still in its infancy, has a promising future. Our work on a retroviral IN represents one possible alternative to achieve these goals, while providing a potential tool for overcoming practical difficulties in transgenic fish technology.

Experimental Procedures

Plasmid construction

MoMLV U3 terminal sequences, 5′ TATGAAAGACCCC 3′ (Bushman and Craigie, 1990), were added to the termini of a dihydrofolate reductase (DHFR) expression cassette by PCR using a TA Cloning Kit (InvitroGen). This modification provided a 43-bp *Nde*I-*Stu*I fragment carrying essential *cis* information for integration (Bushman and Craigie, 1990) and a *Nde*I restriction site for linearization of plasmid pDHFR/IN (from Scott Fahrenkrug). To construct pRSVTk-Neo/IN, the parental pRSVTk-Neo (from Jozsef Szelei) was cut with *Nde*I and filled-in using *E. coli* DNA polymerase I (Klenow fragment), thus eliminating the *Nde*I site in the pBR322 vector. The 43-bp *Nde*I-*Stu*I fragment of pDHFR/IN was blunt-end ligated to the flushed ends of the vector, resulting in an expression construct that could be linearized in the vector backbone with *Nde*I and contained important sequences for integration. A second expression plasmid, pSV2-CAT/IN, was constructed by exchanging the *Acc*I-*Pst*I vector fragment of pSV2-CAT (Gorman et al., 1982) with the respective fragment of pRSVTk-Neo/IN. Thus, pSV2-CAT/IN could also be linearized with *Nde*I and served as a substrate for the MoMLV integrase. For the *in vitro* experiments, pDHFR/IN was cut with *Stu*I to remove most of the expression cassette. In between the *Stu*I sites, a 2,066-bp *Pvu*II-*Eco*RI fragment of pBR322 carrying the tetracycline resistance gene (Tc) was cloned after filling in the *Eco*RI end with Klenow polymerase. *Nde*I digestion of the resultant plasmid, pT6/IN, results in an approximately 2.4-kb fragment containing the Tc gene and retroviral U3 sequences at both ends.

Integrase production, purification, and in vitro assay of activity

The 556-3 recombinant baculovirus expressing the MoMLV integration protein was obtained from Dr Robert Craigie. Large scale production of BV/IN was done essentially as described (Craigie et al., 1990). The *in vitro* integrase reaction was performed essentially as described (Fujiwara and Craigie, 1989), with the following modifications. Circular pUC19 plasmid served as a target, and a 2,400-bp *Nde*I fragment of pT6/IN carrying the tetracycline resistance gene and MoMLV LTR sequences served as specific substrate. The integration reaction was done in 85 mmol/L KCl, 25 mmol/L K-glutamate, 20 mmol/L HEPES (pH, 7.6), 5 mmol/L $MgCl_2$, 5% (v/v) glycerol, 1 mmol/L dithiothreitol, 100 μg/mL bovine serum albumin, and 10% (v/v) dimethyl sulphoxide (including protein storage buffer components). To this solution, 0.2 pmol target DNA, 0.1 pmol substrate DNA, 40 μLMTK$^-$ cell extract, and approximately 5 pmoles BV/IN were added on ice (in ψ-2 experiments, 40 μL ψ-2 cell extract was used as integrase source). Tubes were kept on ice for 1 hour; PEG-8000 was then added to a final concentration of 5% (w/v), and the integration reaction was allowed to proceed at 30°C for 1 hour. Reactions were stopped by the addition of SDS to a final concentration of 0.5% (w/v) and 500 μg/mL Proteinase K, and samples were incubated at 37°C for at least 2 hours, which was followed by phenol-chloroform and chloroform extraction and precipitation with ethanol. DNA was transformed into DH5α cells by electroporation (Biorad), and recombinants were selected on LB plates containing 25 μg/mL tetracycline.

Preparation of cell extracts

All steps of total protein extract preparation (Dignam, 1990) were done on ice. Cells were harvested from confluent plates and centrifuged at 1000 x g for 5 minutes, then washed twice with cold PBS. The cell pellet was suspended in 3 volumes of Buffer C (10 mmol/L Tris-HCl [pH, 7.5], 5 mmol/L $MgCl_2$, 2 mmol/L PMSF) and homoge-

nized in a glass-to-glass homogenizer. Then 1 vol of Buffer D (250 mmol/L Tris-HCl [pH, 7.5], 250 mmol/L KCl, 10 mmol/L $MgCl_2$, 5 mmol/L dithiothreitol, 5 mmol/L PMSF, 0.5 mmol/L EDTA, and 50% [v/v] glycerol) was added during continuous stirring, and the solution was spun at 15,000 x g for 5 minutes. Supernatant was frozen and stored at −70°C in aliquots.

Microinjections

For transient expression experiments pSV2-CAT/IN linearized with *Nde*I as specific substrate of IN at a concentration of approximately 50 μg/mL was injected into the perivitellinar space of early zebrafish embryos at 1 to 2 cell stage. For integration studies, approximately 10^6 copies of pRSVTk-Neo/IN linearized with *Nde*I at a concentration of approximately 50 μg/mL was injected into the cytoplasms of early zebrafish embryos at 1 to 2 cell stage. DNA was mixed with either total protein extracts from LMTK⁻ cells or ψ-2 cells or BV/IN at 0.5 pmol/μL concentration supplemented with LMTK⁻ cell extract and then incubated at 0°C for 10 minutes and at room temperature for 20 minutes prior to injection.

DNA preparation, dot blotting, and hybridization

High molecular weight DNA was prepared from individual fish at 14 days of age. Fish tissues were homogenized in 100 μL of 200 mmol/L Tris-HCl (pH, 8.0), 100 mmol/L EDTA, 150 mmol/L NaCl, 0.5% (w/v) SDS, and 500 μg/mL proteinase K overnight at 55°C. Samples were then extracted once with phenol-chloroform, once with chloroform, and precipitated with 2 vol EtOH. 5 μg fish DNA samples were prepared for blotting by the addition of 1 vol 0.8 mol/L NaOH, incubated at room temperature for 10 minutes; 2 vol ice-cold 2 mol/L ammonium-acetate was then added, and the DNA samples were blotted to a Hybond (Amersham) nylon membrane with the help of a dot-blot apparatus (BRL). Blots were then baked at 80°C for 2 hours and ultraviolet light–irradiated for 30 seconds. A 900-bp *Bgl*II-*Sma*I fragment of pRSVTk-Neo containing the APT-II coding sequences and radioactively labeled with a random priming labeling kit (BRL) was used as a probe in DNA hybridizations, which were carried out according to standard protocols. Autoradiograms were scanned with a Biorad Video Densitometer using a 2D-densitometry software. Statistical analysis of data was done with a Microsoft Excel Version 4.0 software.

CAT assays

Batches of 50 to 60 microinjected fish taken at different times after fertilization/microinjection were homogenized in 250 mmol/L Tris-HCl (pH, 7.5). Assays were done as described previously (Liu et al., 1990) using total protein extracts equivalent to approximately 15 fish. Scintillation counting of cut-out spots on the thin layer chromotography plates was used to determine CAT conversion rates.

PCR analysis

LMPCR was done essentially as described (Mueller and Wold, 1989). Approximately 2 μg genomic fish DNA was first completely digested with *Hae*III restriction endonuclease, a four-base cutter, to produce fragments of several hundred base pairs that could be conveniently amplified by PCR. Then 1 pmol Gene-specific Primer 1 (GSP1) was allowed to anneal to denatured (95°C, 3 min), *Hae*III-digested DNA at 50°C for 30 minutes in 10 μL volume in Sequenase (USB) reaction buffer. Samples were then transferred to ice and 1 μL 0.1 mol/L dithiothreitol, 1 μL of 1 mmol/L dNTPs, and 1.5 μL 1:4 diluted Sequenase (USB) were added. Second-strand synthesis was allowed to proceed at 47°C for 10 minutes, and the reaction was stopped by a 15-minute incubation at 65°C. To this reaction, 3 μL 1 mg/mL bovine serum albumin, 1.5 μL 10 mmol/L ATP, 5 μL 30% (w/v) PEG-8000, 80 pmol PCR linker, and 2 U T4 ligase (BRL) were added to a final volume of 30 μL, and ligation was allowed to proceed overnight at 16°C. DNA samples were then phenol-chloroform and chloroform-extracted and precipitated with EtOH. PCR Linker Primers A and B (LPs A and B) were annealled at equimolar concentrations in 200 mmol/L Tris-HCl (pH, 7.5) and 100 mmol/L NaCl after an initial heating for 3 minutes at 95°C. PCR was performed by a Perkin-Elmer Thermo-Cycler with a 6-minute initial denaturation step at 94°C, then 35 cycles of 1 minute at 94°C, 1 minute at 70°C, and 30 seconds at 72°C. To each reaction, 2 U Vent polymerase (New England Biolabs) and LP A and Gene Specific Primer 2 (GSP 2) to a final concentration of 1 μmol/L were added. Aliquots of PCR reactions were blotted and probed with a 290-bp *Ned*I-*Pvu*II fragment of pRSVTk-Neo/IN. The sequences of oligonucleotide primers were as follows: LP A: 5′-GCGGTGACCCGGGAGATCTGAATT C-3′; LP B: 5′-GAATTCAGATC-3′; GSP 1: 5′-ACCGCAGCTGCCTC GCGCGT-3′; GSP 2: 5′-CCTCTGACACATGCAGCT CCCGG-3′.

Acknowledgments

We thank J.M. Bishop, H.E. Varmus, R. Craigie, K. Mizuuchi, and F. Bushman for discussions; and J. Essner and T. Sonstegard for reading the manuscript. We obtained the 556-3 IN-expressing baculovirus clone and Sf9 insect cells from Dr. Robert Craigie, the ψ-2 packaging cells from Dr. Scott McIvor, the pDHFR/IN construct from Scott Fahrenkrug, and the pRSVTk-Neo plasmid from Dr. Jozsef Szelei. This work was sponsored by Public Health Service (NIH) grant CA43881, USDA grant 92-37205-7842, and grants from the Legislative Committee for Minnesota Resources and the Minnesota Sea Grant College Program (paper JR 305). Supported by the National Oceanographic and Atmospheric Administration, Office of Sea Grant, under grant NOAA-86A-D-SG112, project R/A-6.

References

Brown, P.O. (1990). Integration of retroviral DNA. *Curr Topics Microbiol Immunol* 157:19–48.

Brown, P.O., Bowerman, B., Varmus, H.E., and Bishop, J.M. (1987). Correct integration of retroviral DNA in vitro. *Cell* 49:347–356.

Bushman, F.D., and Craigie, R. (1990). Sequence requirements for integration of Moloney murine leukemia virus DNA in vitro. *J Virol* 64:5645–5648.

Bushman, F.D., and Craigie, R. (1991). Activities of human immunodeficiency virus (HIV) integration protein in vitro: specific cleavage and integration of HIV DNA. *Proc Natl Acad Sci USA* 88:1339–1343.

Chong, S.S.C., and Vielkind, J.R. (1989). Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. *Theor Appl Genet* 78:369–380.

Colicelli, J., and Goff, S.P. (1988). Sequence and spacing requirements of a retrovirus integration site. *J Mol Biol* 199:47–59.

Craigie, R. (1992). Hotspots and warm spots: integration specificity of retroelements. *Trends Genet* 8:187–190.

Craigie, R., Fujiwara, T., and Bushman, F. (1990). The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration in vitro. *Cell* 62:829–837.

Culp, P., Nusslein-Volhard, C., and Hopkins, N. (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc Natl Acad Sci USA* 88:7953–7957.

Dignam, J.D. (1990). Preparation of extracts from higher eukaryotes. *Method Enzymol* 182:194–203.

Does, M.P., Dekker, B.M., de Groot, M.J., and Offringa, R. (1991). A quick method to estimate the T-DNA copy number in transgenic plants at an early stage after transformation, using inverse PCR. *Plant Mol Biol* 17:151–153.

Engelman, A., Mizuuchi, K., and Craigie, R. (1991). HIV-1 DNA integration: mechanism of viral DNA cleavage and DNA strand transfer. *Cell* 67:1211–1221.

Fletcher, G.L., and Davies, P.L. (1991). Transgenic fish for aquaculture. *Genet Eng* 13:331–370.

Fujiwara, T., and Craigie, R. (1989). Integration of mini-retroviral DNA: a cell-free reaction for biochemical analysis of integration. *Proc Natl Acad Sci USA* 86:3065–3069.

Golic, K.G., and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* 59:499–509.

Gorman, C., Moffat, L., and Howard, B. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* 2:1044–1051.

Grandgenett, D.P., and Mumm, S.R. (1990). Unraveling retrovirus integration. *Cell* 60:3–4.

Gridley, T., Soriano, P., and Jaenisch, R. (1987). Insertional mutagenesis in mice. *Trends Genet* 3:162–166.

Gross, D.S., and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. *Annu Rev Biochem* 57:159–197.

Grunstein, M. (1990). Histone function in transcription. *Annu Rev Cell Biol* 6:643–678.

Hackett, P.B. (1993). The molecular biology of transgenic fish. In: *Biochemistry and Molecular Biology of Fishes, vol. 2.* Hochachka, P., Mommsen, T. (eds.). (in press).

Haring, M.A., Rommens, C.M., Nijkamp, H.J., and Hille, J. (1991). The use of transgenic plants to understand transposition mechanisms and to develop transposon tagging strategies. *Plant Mol Biol* 16:449–461.

Ishiura, M., Hirose, S., Uchida, T., Hamada, Y. Suzuki, Y., and Okada, Y., (1982). Phage particle-mediated gene transfer to cultured mammalian cells. *Mol Cell Biol* 2:607–616.

Katz, R.A., Merkel, G., Kulkosky, J., Leis, J., and Skalka, A.M. (1990). The avian retroviral IN protein is both necessary and sufficient for integrative recombination in vitro. *Cell* 63:87–95.

Kaufman, P.D., and Rio, D.C. (1991). Germline transformation of *Drosophila melanogaster* by purified P element transposase. *Nucleic Acids Res* 19:6336.

Kimmel, C.B. (1989). Genetics and early development of zebrafish. *Trends Genet* 5:283–288.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinsky, A.R., and Hackett, P.B. (1990). Development of expression vectors for transient fish. *Bio/Technology* 8:1268–1272.

Mann, R., Mulligan, R.C., and Baltimore, D. (1983). Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* 33:153–159.

Moav, B., Liu, Z., Caldovic, L.D., Gross, M.L., Faras, A.J., and Hackett, P.B. (1993). Regulation of expression of transgenes in developing fish. *Transgen Res* (in press).

Mueller, P.R., and Wold, B. (1989). In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. *Science* 246:780–786.

O'Gorman, S., Fox, D.T., and Wahl, G.M. (1991). Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251:1351–1355.

Orban, P.C., Chui, D., and Marth, J.D. (1992). Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci USA* 89:6861–6865.

Pryciak, P.M., and Varmus, H.E. (1992). Nucleosomes, DNA-binding proteins and DNA sequence modulate retroviral integration target site selection. *Cell* 69:769–780.

Pryciak, P.M., Sil, A., and Varmus, H.E. (1992a). Retroviral

integration into minichromosomes in vitro. *EMBO J* 11:291–303.

Pryciak, P.M., Muller, H., and Varmus, H.E. (1992b). Simian virus 40 minichromosomes as targets for retroviral integration in vivo. *Proc Natl Acad Sci USA* 89:9237–9241.

Rio, D.C., Barnes, G., Laski, F.A., Rine, J., and Rubin, G.M. (1988). Evidence for *Drosophila* P element transposase activity in mammalian cells and yeast. *J Mol Biol* 200:411–415.

Rossant, J., and Hopkins, N. (1992). Of fin and fur: mutational analysis of vertebrate embryonic development. *Genes Dev* 6:1–13.

Roth, M., Tanese, N., and Goff, S. (1988). Gene product of Moloney murine leukemia virus required for proviral integration is a DNA-binding protein. *J Mol Biol* 203:131–139.

Sato, A., Aoki, K., Komura, J., Masahito, P., Matsukuma, S., and Ishikawa, T. (1992). Firefly luciferase gene transmission and expression in transgenic medaka (*Oryzias latipes*) *Mol Marine Biol Biotechnol* 1:318–325.

Sherman, P.A., Dickson, M.L., and Fyfe, J.A. (1992). Human immunodeficiency virus type 1 integration protein: DNA sequence requirements for cleaving and joining reactions. *J Virol* 66:3593–3601.

Shih, C.C., Stoye, J.P., and Coffin, J.M. (1988). Highly preferred targets for retrovirus integration. *Cell* 53:531–537.

Stuart, G.W., McMurray, J.V., and Westerfield, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* 103:403–412.

Stuart, G.W., Vielkind, J.R., McMurray, J.V., and Westerfield, M. (1990). Stable lines of transgenic zebrafish exhibit reproducible patterns of transgenic expression. *Development* 109:577–584.

Varmus, H.E., and Brown, P.O. (1989). Retroviruses. In: *Mobile DNA*. Berg, D.E., and Howe, M.M. (eds.). Washington D.C.: American Society for Microbiology, pp. 53–108.

Vielkind, J.R., and Vogel, K.S. (1989). Gene transfer and expression studies in cultured avian neural crest cells differentiating into melanocytes. *Pigm Cell Res* 2:44–52.

Winkler, C., Vielkind, J.R., and Schartl, M. (1991). Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). *Mol Gen Genet* 226:129–140.

Yoon, S.J., Hallerman, E.M., Gross, M.L., Liu, Z., Schneider, J.F., Faras, A.J., Hackett, P.B., Kapuscinski, A.R., and Guise, K.S. (1990). Transfer of the gene for neomycin resistance into goldfish, *Carassius auratus*. *Aquaculture* 85:21–33.

BLACKWELL/Molecular Marine Biology and Biotechnology 68-N
Final Page No. 173
Page Depth Deviation: −384 pts.

HURON VALLEY GRAPHICS
Record No. 513 File 1

June 25, 1993

LCMR Work Program 1991 Progress Report

I. Cooperative Urban Aquatic Education Program - Fisheries 12

Program Manager: Linda Erickson-Eastwood
Aquatic Education Coordinator
Department of Natural Resources
Section of Fisheries
500 Lafayette Road
St. Paul, MN. 55155
(612) 297-4919

A. M.L. 91, Chpt. 254, Art. 1, Sect. 14, Subd. 8.C

Appropriation:	$340,000
Expenditures:	$340,000
Balance:	$ 0,000

This appropriation is to the commissioner of natural resources to expand urban fishing opportunities and awareness.

II. Narrative

Two-thirds of Minnesota's population resides in urban areas. Much of this population doesn't have access to opportunities for learning about the issues impacting our aquatic resources. Since the future and management of these resources balances upon public awareness and perceptions, a need exists for education regarding these issues. As a cooperative project between DNR, Fisheries Section and Minnesota Extension Service (MES), 4-H Youth Development, this project will in part provide non-formal, experiential and developmental sportfishing opportunities to the urban public (specifically but not limited to youth, females, single parents, elders, and the disabled from a variety of ethnic groups). It will also teach key aquatic concepts and issues. An advisory committee consisting of representatives from key agencies and user groups will provide guidance and direction to this project.

III. Objectives

A. Coordinate Aquatic Education Activities

A1. Narrative: Many agencies and groups within targeted metropolitan areas (including Duluth, St. Paul, and Minneapolis) already have well established educational programs. These agencies and groups can be utilized to reach target audiences.
In addition, representatives from these groups can benefit the program by providing input and review. The focus of this objective will be to solicit representatives for guidance via the MinnAqua Advisory Council. With the aide of this Council, staff will actively seek and establish communication and involvement with individuals, groups and agencies who have established programs.

A2. Procedures: Groups that can benefit and work cooperatively with the program will be identified and prioritized. A staff member will then contact that agency or group to conduct an informational meeting and determine interest and coordinate efforts on a cooperative project. An advisory council consisting of representatives from key groups or agencies interested or involved in aquatic education will meet quarterly to help staff review program progress and materials. They will also help identify other groups or agencies to be contacted about the project and possible alternative funding resources.

A3. Budget
a. Amount Budgeted: $40,000
b. Balance: $ 0.00

A4. Timeline:

	July 91	Jan 92	June 92	Jan 93	June 93
Advisory Meetings	..	..	..	..	..
Id./Pri. Groups	.....	.....			
Material Review		.....		.....	
Program Review	.....	.....		.....	
Contact Agencies	.....	.....	.....	.....	
Secure other funds	.....	.....	.....	.....	

A5. Status: Two MinnAqua Advisory meetings were held in FY 92 (October - May). At the May meeting committee members assisted at the Wilder Clinic. Each participant enjoyed helping teach the youth about their aquatic environments and fishing. The highlight, however, was watching the kids actually fish for the first time. The January meeting was canceled due to scheduling problems. The materials subcommittee reviewed the final draft of the leaders guide. To date the printers are still working on the final layout and will go to print soon.

To foster additional contacts and cooperation, staff will be presenters at the upcoming OEE meeting in Brainerd. Staff were also involved in the OEE planning process and the various meetings held to discuss implementation of the plan. From these meetings, we were asked to work with the seven pilot projects that were selected by the OEE. We have also been asked to present our model program at the upcoming National Association of Extension Service Agents Conference in Kansas City. The program is involved with the National Association of Aquatic Educators which is a part of the AFTMA and Federal Aid support.

Sports Program which teaches youth statewide about Minnesota's aquatic resources which reaches approximately 3,000 youth annually.

This work plan represents an extension and expansion of the program funded in FY 90-91. Semi-annual reports that outline the progress made previously on this project are on file at the LCMR. At the present, no other proposals are being considered for this program.

In the 1991 Legislative session, the DNR secured funds from the Game and Fish Fund ($140,000/year) for the entire DNR MinnAqua Program. These monies were proportioned with 50% going to the 7 county metro area and 50% to outstate areas. These monies will be used to support all six components (Urban Angling, Volunteer Training, Seminars, Displays, Teacher training, and classroom materials) of the MinnAqua Program. Approximately 2/6 of the funds will be used to expand the present LCMR Urban Angling program statewide and in the metro area.

RIM monies have been allocated to support a full-time Aquatic Resource Education Coordinator in DNR to oversee the urban LCMR program as well as develop and coordinate a broader statewide conservation/mgt. oriented MinnAqua Program. RIM monies have also been requested to support the program after LCMR funding ceases.)

D. Cooperative Urban Aquatic Resource Education Program Appropriation: $350,000

E. Cooperative Urban Aquatic Resource Education Program-Fisheries 12
Appropriation: $340,000

VI. Qualifications

1. Program Manager
Linda Erickson-Eastwood
Aquatic Education Coordinator
Section of Fisheries - MnDNR

M.S. Fisheries Biology, University of Wisconsin-La Crosse
Thesis: Computer Aided Analysis of the Backwater Rehabilitation of Weaver Bottoms (Pool 5, UMR): Impacts on Nursery Habitat. In addition to my thesis work, I worked 4 1/2 years as an employee of the Missouri Department of Conservation. Specific duties included coordinating educational activities, providing public relations for fisheries activities, and helping develop and review materials used by MDC, other agencies and groups.

2. Major Cooperators:
A. Steve Bilitz
Aquatic Resource Educator
Minnesota Extension Service 4-H Youth Development
Natural Resource Programming

M.S. Elementary Education, Eastern Michigan University, Ypsilanti, MI. He was a teacher for 15 years working with elementary, junior high and high school youth. He has also worked with In-Fisherman's Communications Network and Camp Fish for 3 years. He then contracted with DNR Fisheries Section to do an Aquatic Education Needs Assessment. After completing this report he became MES's program leader for this project.

B. Peter Jacobson
Education Specialist
Department of Natural Resources
Section of Fisheries

B.S. Education, St. Cloud State University, St. Cloud, MN. 1990
B.S. Agronomy, University of Minnesota-St. Paul, 1987
He has been very active in 4-H programs throughout his lifetime. Was a science teacher in South Dakota when hired on for this program. Also was a student teacher for the Monticello High School. Is a professional fishing guide for the Mississippi River Valley.

C. Mary F. Kroll
Aquatic Education Coordinator
Minnesota Extension Service
4-H Youth Development

B.S. Forest Resources, University of Minnesota College of Forestry
Has done extensive work in developing and writing resource oriented materials for MES and other agencies.

VII. Reporting Requirements
Semiannual status reports will be submitted not later than January 1992, July 1992, January 1993 and a final report by June 30, 1993.

Activity Summary
Fisheries' L.C.M.R. Proposal
Cooperative Urban Aquatic Research Education Program
January 1, 1993 - June 18, 1993

Regions 1, 2, & 3

	# of Events	# of Participants
Special Events	13	528
Clinics	4	356
Nibbles	21	1414
Volunteers	10	78
Grand Total	**48**	**2,376**

ETHNIC STATISTICS

	Special Events	Clinics	Nibbles	Trainings	Total
AA =	1	0	2	0	3
BL =	7	0	4	0	11
C =	202	80	286	36	604
H =	0	0	0	0	0
NA =	20	0	9	0	29
UNK =	298	276	1113	42	1729

GENDER

	Special Events	Clinics	Nibbles	Trainings	Total
M =	405	147	689	57	1298
F =	123	209	725	21	1078

Activity Summary
Fisheries' L.C.M.R. Proposal
Cooperative Urban Aquatic Research Education Program
January 1, 1993 - June 18, 1993

SPECIAL EVENTS: An event that is less than 6 hours that includes classroom and hands-on experiences.

County Program ID	Total	Gender		Ethnic Group					
		M	F	AA	BL	C	H	NA	UNK
Becker									
Tamarack Wild Ref,6/5	15	10	5						15
Cook									
Grand Marais, 5/27	56	23	23		1	53		2	
Crow Wing									
Park & Rec, 6/11	3	1	2			3			
Itasca									
Murphy Elem, 3/30	47	30	17			47			
Comm Ed, 6/7	6	5	1			3		3	
Lake									
Silver Bay Elem, 5/27	52	25	27			52			
St. Louis									
YWCA Ice Fish, 2/20	17	7	10	1	5	8		3	
YMCA Ice fish, 2/27	15	12	3			2			13
YWCA, 5/22	39	17	22		1	28		10	
Comm Ed, 6/8	8	5	3			6		2	
Stearns									
Boy Scouts, 6/14-16	270	270							270
Total Events: 13 #:	**528**	**405**	**123**	**1**	**7**	**202**	**0**	**20**	**298**

CLINICS: An event that included a minimum of 6 hours of classroom and hands-on experiences resulting in a certificate.

County Program ID	Total	Gender		Ethnic Group					
		M	F	AA	BL	C	H	NA	UNK
Aikin									
County Day Camp, 6/15	56	32	24						56
Carlton									
Girl Scouts, 6/14	38		38			38			
Girl Scouts, 6/15	42		42			42			
Crow Wing									
TAF Park & Rec, 6/12	220	115	105						220
Total Clinics: 4 #:	**356**	**147**	**209**			**80**			**276**

NIBBLES: An event that informs a group about the MinnAqua Program and identifies people interested in clinics or training sessions.

County Program ID	Total	Gender		Ethnic Group					
		M	F	AA	BL	C	H	NA	UNK
Becker									
4-H Leaders, 1/22	7	4	3			7			
Soil/wat cons day,5/10	232	115	117						232
4-H, 6/1	29	13	16						29
4-H, 6/2	29	13	16						29
Clearwater									
4-H, 6/5	16	8	8			8			
Crow Wing									
4-H Leaders, 1/8	10	5	5			10			
Bassmasters, 3/8	20		20			20			
MAVA, 3/26	7	2	5			7			
Baxter Elem, 5/27	120	50	70						70
Douglas									
TAF Viking S.C., 6/12	68	40	28						68
Hubbard									
4-H, 5/24	96	40	56						96
Itasca									
Blandin Found., 4/24	85	42	43	1	2	78		5	
TAF DNR, 6/12	49	34	15						49
West Ottertail									
4-H, 5/8	57	14	43						57
Norman									
4-H, 5/11	139	60	79						139
Polk									
TAF Erskine, 6/12	122	87	35						122
St. Louis									
USFS, 5/5	120	60	60		1	118		1	
TROUT, 6/2	24	12	12						24
TROUT, 6/3	127	60	67						127
Park & Rec, 6/10	22	10	12		1				21
TAF YMCA, 6/12	35	20	15	1		31		3	
Total Nibbles: 21 #:	**1414**	**689**	**725**	**2**	**4**	**286**	**0**	**9**	**1113**

TRAININGS/VOLUNTEERS: Individuals trained as program instructors or helpers, or sponsor doing support activities.

County Program ID	Total	Gender		Ethnic Group					
		M	F	AA	BL	C	H	NA	UNK
Crow Wing									
RSVP, 3/17	7	1	6			7			
RSVP, 4/6	6	1	5			6			
Comm Service, 6/2	10	10							10
Comm Service, 6/3	10	10							10
Comm Service, 6/4	12	12							12
VH Park & Rec, 6/10	2		2			2			
Vol. Inst. 6/15	9	5	4			9			
Lake of the Woods									
Baudette Elem, 5/25	9	9							9
St. Louis									
UMD Outdoor, 5/26	4	3	1			4			
Laurentian EE, 6/11	9	6	3			8			1
Total 10 #:	**78**	**57**	**21**			**36**			**42**

Activity Summary - Regions 4,5, & 6
Fisheries' L.C.M.R. Proposal #12
Cooperative Urban Aquatic Research Education Program
January 1 - June 18, 1993

	# of Events	# of Participants
Special Events	33	3,505
Clinics	17	981
Nibbles	16	3,187
Volunteer Trainings	4	42
Grand Total	**70**	**7,715**

Activity Summary - Regions 4,5, & 6
Fisheries' L.C.M.R. Proposal #12
Cooperative Urban Aquatic Research Education Program
January 1 - June 18, 1993

Ethnic Statistics

Special Events		
AA	=	37
BL	=	12
C	=	1,062
H	=	62
NA	=	0
UNK	=	2,332
TOTAL	**=**	**3,505**

Clinics		
AA	=	142
BL	=	103
C	=	295
H	=	25
NA	=	147
UNK	=	269
TOTAL	**=**	**981**

Nibbles		
AA	=	6
BL	=	10
C	=	1,764
H	=	5
NA	=	1
UNK	=	1,401
TOTAL	**=**	**3,187**

Volunteer Trainings		
AA	=	1
BL	=	0
C	=	31
H	=	0
NA	=	0
UNK	=	10
TOTAL	**=**	**42**

ACTIVITIES UPDATE - REGIONS 4,5, & 6
FISHERIES' L.C.M.R. PROPOSAL #12
COOPERATIVE URBAN AQUATIC RESEARCH EDUCATION PROGRAM
JANUARY 1 - JUNE 18, 1993

*Codes: AA = Asian American BL = African American C = Caucasian H = Hispanic
NA = Native American UNK = Unknown N/M = Not Measured N/A = Not Available

SPECIAL EVENTS: An event that is less than 6 hours that includes classroom and hands-on experiences or a one day event that is not a structured clinic.

			*Gender		*Ethnic Group					
County	Program ID	Total	M	F	AA	BL	C	H	NA	UNK
Anoka	Fridley Community Ed 4/14,21	15	13	2			15			
	Springbrook Nature Ctr. 5/4-7	270	135	135						270
	Centerville Elem. #1 6/2	17	13	4			17			
	Centerville Elem. #2 6/3	44	28	16				1		43
Fillmore	Lanesboro Sp. Fish Day 6/2,3	200	N/M	N/M						200
Hennepin	Fish Fair '93 4/13	1500	N/M	N/M						1500
	MW Mountaineering Care Fest 4/24	10	4	6			10			
	McKnight Early Education Ctr. 6/2	61	N/M	N/M						61
	Cub Scout Pack 283 6/5	37	29	8			37			
	Hillcrest Community School 6/7	29	15	14	1		26	1		1
	Nicollet Early Learning 6/11	15	7	8	3	6	6			
Kandiyohi	Spicer Environmental Days 5/4-6	600	300	300	6	3	531	60		
Le Sueur	St. Peter Cub Scouts 4/26	40	32	8			40			
McLeod	Lynn Town Hall 4/12	45	18	27			45			
Mower	Lake Louise State Park 6/5	12	7	5			12			
Nicollet	St. Peter Community Ed. #2 6/18	7	6	1			7			
Olmsted	Rochester Park & Rec 6/14,16	9	N/M	N/M			9			
Ramsey	Fairview Community Ed 2/25	22	21	1			22			
	Ramsey Cub Scouts 3/7	14	13	1			14			

Special Events (continued)

Ramsey	Roseville Middle Schools 4/22	120	60	60						120
	McDonough 4-H Club #1 5/3	15	9	6	15					
	Tiger Cubs 5/16	96	72	24	2	1	93			
	McDonough 4-H Club #2 6/16	10	9	1	10					
Rice	Rice County Sports Group 4/1	45	33	12						45
	Northfield 6/11	N/A								
Scott	Credit River Comets 4-H 5/17	27	10	17			27			
Sherburne	Talihi Elementary 1/25	145	110	35		2	143			
Washington	Stillwater Pack 375 2/3	8	8				8			
Waseca	Waseca Community Ed. 6/11	13	N/M	N/M						13
Watonwan	St. James Library 6/14	43	N/M	N/M						43
	Madelia Library 6/14	21	N/M	N/M						21
Winona	Winona Community Ed. 6/8	15	6	9						15
	Lake Winona 6/15	N/A								
TOTALS:	**# OF SPECIAL EVENTS = 33**	**3505**	**958**	**700**	**37**	**12**	**1062**	**62**	**0**	**2332**

CLINICS: An event that includes a minimum of 6 hours of classroom and hands-on experiences resulting in a certificate.

			*Gender		*Ethnic Group					
County	Program ID	Total	M	F	AA	BL	C	H	NA	UNK
Carver	Watertown Community Ed. 6/4	22	20	2			22			
Dakota	Lakeville Girl Scouts 2/7	14	3	11			14			
	Rosemount Middle School 5/5...	20	18	2			20			
	Scott Highland Sch. 5/11,13,15	20	13	7			20			
	Burnsville Community Ed. 5/22	30	18	12		7	18	5		
Hennepin	Wilder Schools 5/10,13,14,18,20,21	240	120	120						240
	Webster Open School 5/7,14,21	20	17	3	8	3	9			
	Osseo Jr. High 5/20	52	47	5	1	1	50			
	Richfield Home Education 6/4	25	19	6						
	Mpls Schools - Lincoln 6/8	20	10	10	6	8	6			
	Seward Montessori School 6/8	22	16	6	1	4	17			
Ramsey	Maplewood Nature Ctr 2/11,13	40	24	16			40			
	Baptist School 5/3-6	36	20	16	2		33	1		
	Como Elementary #1 5/3-6	101	60	41	31	22	8	1	35	4
	Como Elementary #2 5/10-14	134	75	59	42	23	14	7	48	
	Como Elementary #3 5/17-21	76	42	34	24	11	11	4	26	
	Como Elementary #4 5/24-28	109	61	48	27	24	13	7	38	
TOTALS:	**# OF CLINICS = 17**	**981**	**583**	**398**	**142**	**103**	**295**	**25**	**147**	**269**

NIBBLES: An event that informs a group about the **MinnAqua Program** and identifies people interested in clinics or training sessions.

			*Gender		*Ethnic Group					
County	**Program ID**	**Total**	**M**	**F**	**AA**	**BL**	**C**	**H**	**NA**	**UNK**
Anoka	Champlin High School 4/24	234	126	108			231			3
	Carp Fest 6/13	171	N/M	N/M						171
Blue Stone	Wheaton/Clinton 4th Graders 5/26	89	45	44	1		87		1	
	Ortonville Elementary 5/27	53	27	26						53
Brown	MN Bass Federation 4/3	120	60	60						120
Dakota	MN Polebenders Association 2/15	20	20							20
Goodhue	Zumbrota High School 2/27	225	205	20			225			
Hennepin	Earth Expo, Shingle Creek 4/15	N/A								
	Panfish Kickoff 5/8	158	N/M	N/M			158			
Ramsey	Sportsman's Show 1/23,24	940	N/M	N/M			893			47
	Sportfishing Congress 2/27	100	50	50						100
	Kid Fest 5/16	25	N/M	N/M			25			
	TAK Fishing 6/12	800	N/M	N/M	5	10	48	5		732
	YLE Water Resources 6/16	50	25	25			45			5
Scott	Cedar Lake Assn. 3/20	52	32	20			52			
Winona	TAK Fishing-Rochester 6/12	150	N/M	N/M						150
TOTALS:	**# OF NIBBLES = 16**	**3187**	**590**	**353**	**6**	**10**	**1764**	**5**	**1**	**1401**

VOLUNTEER TRAININGS: An event that trains individuals as program instructors or helpers.

			*Gender		*Ethnic Group					
County	**Program ID**	**Total**	**M**	**F**	**AA**	**BL**	**C**	**H**	**NA**	**UNK**
Brown	New Ulm Training 4/6	15	14	1			15			
Hennepin	Eastman Nature Center 2/5,6	10	9	1						10
	Program Intern Training 4/28-30	17	9	8	1		16			
Rice	Northfield Training 6/9-11	N/A								
TOTALS:	**# OF TRAININGS = 4**	**42**	**32**	**10**	**1**	**0**	**31**	**0**	**0**	**10**

MinnAqua Activity Calendar 1993
Regions 1, 2, and 3

5/27/93

January

Region	Day	Type	Program ID	Number	Time	Contact
3	8	Nibble	4-H Cluster 9	10	10:00	Linda B.
1	22	Nibble	4-H Cluster 7	7	12:00	Linda B.

February

Region	Day	Type	Program ID	Number	Time	Contact
2	20	Clinic	YMCA			Deb L.
2	27	Clinic	YWCA			Deb L.

March

Region	Day	Type	Program ID	Number	Time	Contact
3	8	Nibble	Baxter Bassmaters	20	7 pm	Linda B.
3	17	Sponser	RSVP Stuffing	7	9 - 4	Linda B.
3	26	Training	MAVA	7	1 - 2:15	Linda B.

April

Region	Day	Type	Program ID	Number	Time	Contact
3	6	Sponser	RSVP Stuffing	7	9-3	L. Braun
2	24	Nibble	Earth Day			Deb. L.

May

Region	Day	Type	Program ID	Number	Time	Contact
1	10	Nibble	Becker Cons. Days	232	9-3	Dick F.
1	11	Nibble	Norman County EE Classes	139	9-2	Dick F.
2	22	Event	YWCA Chambers Grove		1-4	Deb L.
1	24	Nibble	Park Rapids			Dick F.
1	25	T. Training	Baudette InService		8-4:30	Linda B.

2	26	V.Training	Sports & Health Center		9-3	Deb. L.
2	27	Event	Silver Bay Elem		9-11	Deb L.
2	27	Event	Grand Marais Elem		1-3	Deb L.

June

Region	Day	Type	Program ID	Number	Time	Contact
1	1,2	Clinic				
2	2	Event	YMCA/FBA/MN Power		5-7pm	Deb L.
1	5	Event	Tamarack W.R.			Dick F.
2	7	Event	Comm Ed #316		1-3	Deb L.
2	8	Event	Comm Ed Floodwood		1-3	Deb L.
2	9	Nibble	Duluth Park & Rec			Deb
2	10	Event	Comm Ed #316		1-3	Deb
3	11	Event	Brainerd Seniors Fest.		10-2	Linda B./AD
	12	Clinic	Brainerd Park & Rec		9 - 3	Linda B./AD
1	12	TAF	Erskine(OPEN)			Dick F.
2	12	TAF	Blandin Beach Grand Rapids(OPEN)		9-12	Deb
2	12	TAF	YMCA/FBA/Kiwanis Club		8:30-2	Deb
2	14	Clinic	Northern Pines Girl Scout		8:30-2	Deb
3	14,15,16		St. Cloud Day Camp			Linda B./AD
3	15&16	V.Training	Brainerd		6-9pm	LB/AD/JC
3	15	Clinic	Cass Co 4-H			Linda
2	15	Clinic	Northern Pines Girl Scout		9-3	Deb
3	17	Clinic	Cass Co 4-H			Annette
2	17	Clinic	Congdon/Tischer Comm Center(OPEN)		10-4	Deb
	19		Clearwater 4-H			Dick F.
2	19	Event	Grand Rapids Park & Rec(OPEN)		10-12	Deb
1	21	V.Training	Stevens Co 4-H			Linda B./Dick F.

1	23	Clinic	Stevens Co		Dick F.
2	24	Nibble	CityWide Fishing Content (OPEN)	11:30-2:30	Deb
2	25	V.Training	Kawishiwi Pavillion,Ely	9-3:30	Deb
3	25	Nibbles	Browerville Days		Annette
3	26	Clinic	Heritage Nature Center		Annette
3	27	Clinic	Heritage Nature Center		Annette
2	28	Clinic	Courage Center, Duluth	12-5	Deb
1	30	Clinic	Wadena Camp Courage	9-3	Dick F.

July

Region	Day	Type	Program ID	Number	Time	Contact
3	1	Clinic	Cass Co 4-H			Annette
3	6,7,8	Clinic	Mille Lacs Camp			Annette
2	8	Clinic	Boys & Girls Club, Part 1		1-4	Deb
2	13	Clinic	Comm Ed, Cook		9-3	Deb
2	14	Event	Chester Bowl Ski (OPEN)		1-3	Deb
2	15	Clinic	Boys & Girls Club, Part 2		12-3	Deb
1	15		Wheeler 4-H			Dick F.
1	19		Fergus Falls 4-H			Dick F.
2	19	Event	Cub Scouts Day Camp			Deb
2	20	Event	Cub Scouts Day Camp			
2	28	Clinic	Comm Ed Cook		9-3	Deb
3	28	Clinic	Cass Co 4-H			Annette

August

Region	Day	Type	Program ID	Number	Time	Contact
1	4		Clay Co 4-H			Dick F.
3	4		Wadena Club Scouts			Annette D.

3	4		Wadena Club Scouts		Annette D.
1	6		Ottertail 4-H		Dick F.
1	10		Pennington 4-H		Dick F.
3	11,12	Event	Boy Scouts		Annette D.
2	12	Event	Outdoor Program Day Camp		Deb
1	17		Becker 4-H		Dick F.
2	20	Nibble	Itasca Co Fair	1-4	Deb
2	21	Nibble	Itasca Co Fair	1-4	Deb

MinnAqua Activity Calendar 1993

Version: June 16, 1993

Regions 4, 5, & 6

JANUARY

DATE	TYPE	PROGRAM ID	TIME	CONTACT
8,9 C	Clinic	Lino Lakes Ice fishing clinic	cancelled	A.Drewes
10 C	Clinic	St.P Park & Rec Winter Fest ice fishing	cancelled	A.Drewes
23,24	Nibble	Sportsman's Show, Ramsey Co	11:00a-1:30p	L.E.Eastwood A.Drewes
25	Event	Talihi Elementary, Sherburn Co.	7:00p	L.E.Eastwood
25	Nibble	Scout Meeting	cancelled	L.E.Eastwood G.Leach

FEBRUARY

DATE	TYPE	PROGRAM ID	TIME	CONTACT
3	Event	Stillwater Pack 375, Wash Co	6:30p-8:00p	L.E.Eastwood T.Schlagenhoft
5,6	V.Training	Eastman Nature Ctr, Henn Co	6:00p-9:00p 9:30a-3:30p	A.Drewes
7	Clinic	Lakeville Girlscouts Ice fishing		S.Patterson
11,13	Clinic	Maplewood Nature Ctr ice fishing	6:30p-9:00p 3:30p-5:30p	A.Hutchinson S.Patterson
20		Volunteer Stuffing Party	cancelled	S.Patterson
25	Event	Fairview Community Ed, RAM Co.		L.E.Eastwood
27	Nibble	MN Sportfishing Congress, Shoreview	11:00a-11:30a	J.Schneider
27	Nibble	Zumbrota High School, GO Co.		L.E.Eastwood

MARCH

DATE	TYPE	PROGRAM ID	TIME	CONTACT
7	Event	Ramsey Cub Scouts	12:30pm-3:30pm	R.Masanz
18,25,1	Clinic	Maplewood Fishing Clinic	cancelled	I.Howard
20	Nibble	Cedar Lake Association		L.E.Eastwood

MinnAqua Activity Calendar 1993

Version: June 16, 1993

APRIL

DATE	TYPE	PROGRAM ID	TIME	CONTACT
1	Event	Rice County Sports Group		S.Patterson
5,6	Clinic	Poplar Bridge	cancelled	M.Kroll
6	Training	New Ulm Training		L.E.Eastwood
12	Event	Lynn Town Hall		C.Kavanaugh
13	Event	Fish Fair 93		L.E.Eastwood
14,21	Event	Fridley Comm. Ed. Center		S.Patterson
15	Event	Working Parent Resource Network	cancelled	M.Kroll
15	Nibble	Earth Expo, Shingle Creek		L.E.Eastwood
22	Event	Roseville Middle Schools	8:00a-12:00p	M.Kroll
23,30, May 7,14,21	Clinic	Webster Open School, HENN Co.		B.Holden
24	Event	MW Mountaineering Care Fest		P.Yang
24	Nibble	Champlin High School		T.Stanton
26	Event	St.Peter Cubscouts		M.Kroll
28-30	Training	MinnAqua Program Intern Training		M.Kroll
29	???	Volunteer Barbecue		

MAY

DATE	TYPE	PROGRAM ID	TIME	CONTACT
3	Event	Spoon Lake, RAM	4:00p-6:30p	P.Yang
3-5,6	Clinic	Baptist School	2:00p-2:55p 11:00a-2:30p	S.Patterson
3-5,6	Clinic	Como Elementary #1 5th grade	8:00a-11:00a 12:45p-2:15p 11:00a-2:30p	A.Drewes
4-7	Event	Springbrook Nature Center, ANK Co.	8:00a-4:00p	M.Kroll

MinnAqua Activity Calendar 1993

Version: June 16, 1993

MAY (cont'd)

DATE	TYPE	PROGRAM ID	TIME	CONTAC
4-6	Event	Spicer Environmental Days		D.Peterson
5,12,19, 26,June2	Clinic	Rosemount Middle Schools *	3:45p-5:15p	P.Yang
8	Nibble	Panfish Kickoff		S.Patterson
8	Event	Stillwater Boy Scouts	cancelled	P.Yang
10-13,14	Clinic	Como Elementary #2	8:00a-11:00a 12:45p-2:15p 11:00a-2:30p	A.Drewes
10,13-14,18 ✓ 20,21	Clinic	Wilder School	8:30a-12:00p 9:30a-11:30a 12:00p-2:00p	M.Kroll S.Patterson P.Wieczorek
11 ✓/R	???	Program Intern Teleconference	Morning	M.Kroll/A.Drewes All Staff
11,13,15	Clinic	Scott Highland Middle School *	6:30p-8:30p 9:00a-12:00p	P.Yang
14,21,22	Clinic	Forest Lake School	cancelled	P.Yang
16	Nibble	Kid Fest		L.E.Eastwood
16	Event	Tiger Cubs	1:00p-3:30p	R.Masanz P.Wieczorek
17-20,21	Clinic	Como Elementary #3	8:00a-11:00p 12:45p-2:15p 11:00a-2:30p	A.Drewes
20	Clinic	Osseo Jr. High, Hennepin Co	9:00a-2:00p	L.E.Eastwood
22	Clinic	Burnsville Community Ed.,DAK		L.E.Eastwood P.Yang
24-27,28	Clinic	Como Elementary #4	8:00a-11:00a 12:45p-2:15p 11:00a-2:30p	A.Drewes
26	Nibble	Wheaton & Clinton 4th Graders, BS Co.	9:00a-2:00p	J.Kingsley
27	Nibble	Ortonville Elem., BS Co.	9:00a-2:00p	J.Kingsley
27	Event	JD Rivers Planting Festival	cancelled	L.E.Eastwood B.Brekke

MinnAqua Activity Calendar 1993

Version: June 21, 1993

JUNE

DATE	TYPE	PROGRAM ID	TIME	CONTACT
1 M/A	Meeting	Program Interns @ St.Paul	10:00am-3:00pm	L.Braun All Staff
2 ✓	Event	Centerville Elem. #1, ANK Co.	9:30a-2:45p	L.E.Eastwood
2 ✓	Event	McKnight Early Education Ctr. HENN Co.	10:30a-2:30p	S.Patterson
2,3 ✓	Event	Lanesboro Special Fishing Day	9:30am	P.Ruen P.Markowitz
3 ✓	Event	Centerville Elem. #2, ANK Co.	9:30a-2:45p	L.E.Eastwood
4 ✓	Clinic	Richefield Home Education	9:00a-3:00p	S.Patterson
4 ✓	Clinic	Watertown Community Ed.		P.Wieczorek
5 ✓	Event	Cub Scout Pack 283, HENN Co.	10:00a-12:00p	S.Patterson
5 ✓	Event	Lk. Louise St. Park, MOW Co.	1:00p-4:00p	J.Groth
7 ✓	Event	Hillcrest Community Ed.		A.Drewes
8 ✓	Clinic	Seward Montessori, HENN Co.	9:00a-3:00p	P.Wieczorek
8 ✓	Clinic	Mpls. Schools--Lincoln, HENN Co.	8:00a-3:00p	S.Patterson
8 ✓	Event	Winona Community Ed, WIN Co.	8:00a-1:00p	J.Groth
9-11	Training	Northfield	6:00p-9:00p	J.Groth
11	Event	Northfield	6:00p-9:00p	J.Groth
11 ✓	Event	Nicollet Early Learning, HENN Co.	9:30a-12:00p	S.Patterson
11 ✓	Event	Waseca Community Ed., WAS Co.	9:00a-11:00a	D.Peterson
12 ✓	~~Event~~ Nibble	US F&W, Prairie Island, WIN Co.	8:00a-1:00p	J.Groth
~~12~~	Nibble	TAK Fishing, Winona	7:00a-3:00p	J.Groth
12	Nibble	TAK Fishing		Twin Cities Staff
13 ✓	Nibble	Carp Fest		Twin Cities Staff
14 ✓	Event	St. James Library, WAT Co.	10:00a,2:00p	D.Peterson
14 ✓	Event	Madelia Library, WAT Co.	2:00p-	D.Peterson
14,15,29	Clinic	Sibley Manor 4-H Club, RAM Co.	2:00p-4:00p	P.Yang
14,16 ✓	Event	Rochester Park & Rec #1, OL Co.	9:30a-11:30a	J.Groth

MinnAqua Activity Calendar 1993

Version: June 21, 1993

JUNE (cont'd)

DATE	TYPE	PROGRAM ID	TIME	CONTAC
15	Event	Lake Winona #1	9:00a-12:00p	J.Groth
15	Event	Lake Winona #2	12:00p-3:00p	J.Groth
16	Clinic	St.Peter Community Ed., NI Co.	9:00a-3:00p	D.Peterson
16 ✓	Event	YLE Water Resources, S.Carlson		A.Drewes
16 ✓	Event	McDonough 4-H Club #2, RAM Co.	9:30a-12:00p	P.Yang
18	Clinic	St. Peter Community Ed., NI Co.	9:00a-3:00p	D.Peterson
19	Nibble	Lk. Hanska Sportsmen, BR Co.	10:00a-12:00p	D.Peterson
19	Event	Albert Lea	1:00p-3:00p	J.Groth
21	Clinic	Harrison Park		P.Wieczorek
22	Event	Coon Rapids Dam	9:15am-12:00pm	S.Patterson
22	Clinic	Elliot Park		P.Wieczorek
22	Clinic	Osseo		S.Patterson
22,23	Training	Austin	1:00p-3:00p	J.Groth
22,24	Nibble	St.Peter Community Ed., NI Co.	9:00a-3:00p	D.Peterson
23	Event	Rice County Ext.	9:00a-2:30p	A.Drewes
23	Event	Jackson Co. Ext., Lake Shetek	9:30a-5:30p	D.Peterson
24	Event	Camp Sacajawela, DAK	9:30a-1:30p	A.Drewes
25	Event	St. Paul Special Ed., RAM Co.	6:30p-8:30p	A.Drewes
26	Event	RedRock Sportsmen, COT Co.	10:00a-2:00p	D.Peterson
26	Event	Woodbury, WASH Co.	9:00a-12:00p	P.Yang
28	Event	4-H Camp, CH Co.	3:00p-8:00p	D.Peterson
28-Jul 2	Clinic	4-H Camp, YM Co		D.Peterson
29	Meeting	Teleconference	Morning	A.Drewe
30	Event	Byron Park & Rec	9:30a-12:00p	J.Groth
30	Event	Fairmont Community Ed. #1, MART Co.	cancelled	D.Peterson
30	Clinic	Martin Luther King Park		P.Wieczorek P.Yang

MinnAqua Activity Calendar 1993

Version: June 21, 1993

JULY

DATE	TYPE	PROGRAM ID	TIME	CONTACT
1	Clinic	North Commons Park		P.Wieczorek
1	Event	Swift Co. Ext., CH Co.	10:00a-12:00p	D.Peterson
1	Event	Lake Winona #3	4:00p-5:30p	J.Groth
6	Event	Lake Kohlmeir #1	9:00a-4:00p	J.Groth
6,7	Event	New Ulm Library, BR Co.	2:00p-3:00p	D.Peterson
6-8	Event	Northwest YMCA, RAM Co.	12:30p-2:30p	P.Yang
7	Event	Camp Winnebago #1	10:00a-12:00p	J.Groth
8	Clinic	Olivia, REN Co.		D.Peterson
8	Clinic	Bethune Park		A.Drewes P.Wieczorek
8	Clinic	4-H Camp, Paynesville, Meeker Co		D.Peterson
10	Event	Camden State Park, LY Co.	1:00p-4:00p	D.Peterson
10	Clinic	Thomas Lk Pavillion, Eagan, DAK	9:30a-4:00p	J.Storland
12	Event	Worthington Community Ed., NO Co.	12:00p-4:00p	D.Peterson
12,14	Clinic	Mounds Park All Nations, RAM	12:30p-3:30p	A.Drewes
13	Event	Fairmont Community Ed. #2, MART Co.	10:00a-3:00p	D.Peterson
13	Clinic	Luxton Park		P.Wieczorek P.Yang
14	Event	Action Program	1:00p-4:00p	J.Groth
14,16	Clinic	Rochester Park & Rec #2	9:00a-12:00p	J.Groth
15	Clinic	Bottineau Park		P.Wieczorek
15	Event	Lac Qui Parle St. P., LQP Co.	1:00p-5:00p	D.Peterson
15	Event	Lake Kohlmeir #2	9:00a-4:00p	J.Groth
17	Clinic	Ortonville, BS Co.	10:00a-3:00p	D.Peterson
17	Event	Oxbow Park	1:00p-3:00p	J.Groth
20	Event	Swift County Ext. #2, SW Co.	10:00a-12:00p	D.Peterson
20	Event	St. Charles Park & Rec	1:00p-5:00p	J.Groth

MinnAqua Activity Calendar 1993

Version: June 21, 1993

JULY (cont'd)

DATE	TYPE	PROGRAM ID	TIME	CONTACT
21	Event	Luverne 4-H, Rock Co.	9:00a-12:00p	D.Peterson
21	Event	Winona Housing Authority	12:00p-3:00p	J.Groth
22	Clinic	Phelps Park		A.Drewes P.Wieczorek
23	Clinic	Wilmar, KAN Co.	cancelled	D.Peterson
23	Event	Caledonia Community Ed.	9:00a-12:00p	J.Groth
24	Clinic	Cub Scout Pack 626, RAM Co.	9:00a-3:00p	P.Yang
26	Event	PRO New Ulm, BR Co.		D.Peterson
27	Event	Camp Winnebago #2	10:00a-12:00p	J.Groth
28	Event	Swift Co. Ext #3, SW Co.	10:00a-3:00p	D.Peterson
28	Clinic	Stewart Park		P.Wieczorek
29	Clinic	Fairview Park		P.Wieczor...
29	Event	Waseca Community Ed #2	9:00a-11:00a	D.Peterson
29	Event	Plainview Park & Rec #1	9:00a-12:00p	J.Groth
31	Event	Mankato YMCA, LS Co.	9:00a-12:00p	D.Peterson

MinnAqua Activity Calendar 1993

Version: June 21, 1993

AUGUST

DATE	TYPE	PROGRAM ID	TIME	CONTACT
3	Clinic	Matthews Park		A.Drewes P.Wieczorek
3	Event	New Ulm Girl Scouts, BR Co.	12:00p-3:00p	D.Peterson
4	Clinic	Central Park		P.Wieczorek
4	Event	Lake City		J.Groth
4,6	Clinic	Watonwan County Ext., WAT Co.	9:00a-3:00p	D.Peterson
6	Event	Faribault Park & Rec	9:00a-12:00p	J.Groth
9	Event	Fairmont Community Ed. #3, MART Co.	10:00a-3:00p	D.Peterson
10	Clinic	Phillips Park		P.Wieczorek
10	Event	Winona Day Camp	12:00p-4:00p	J.Groth
10,11	Nibble	Ortonville, BS Co.		D.Peterson
11	Event	Camp Courage	9:00a-12:00p	J.Groth
12	Nibble	Chippewa County Fair, CH Co.		D.Peterson
12	Event	Camp Winnebago #2	10:00a-12:00p	J.Groth
14	Event	Camden State Park, LY Co.	1:00p-4:00p	D.Peterson
15	Event	Faribault Co Ext	1:00p-5:00p	D.Peterson
18	Clinic	Powderhorn Park		P.Wieczorek P.Yang
18	Event	LeSueur Community Ed., LS co.	1:00p-5:00p	D.Peterson
19	Event	Plainview Park & Rec #2	9:00a-12:00p	J.Groth
26-7	Nibble	State Fair		A.Drewes All Staff
30 or 31	Meeting	Conference @ St.Paul		A.Drewes All Staff

July 1, 1993

LCMR Final Status Report - Summary - Research

I. Aquaculture Facility Purchase and Development and Transgenic Gamefish Growth Studies - Fisheries 24

Program manager:
Ira R. Adelman
Department of Fisheries and Wildlife
College of Natural Resources
University of Minnesota
1980 Folwell Avenue
St. Paul, MN 55108
612-624-3600

A. M.L. 91 Ch __ Sec. __ Subd: 8 (b) Appropriation: $1,200,000
Balance: $0

Aquaculture Facility Purchase and Development and Genetic Gamefish Growth Studies: This appropriation is to the University of Minnesota, College of Natural Resources, to acquire and develop an aquaculture facility and to continue research on genetically engineered gamefish.

B. Compatible Data: NA

C. Match Requirement: $364,400 from the University of Minnesota

II. Narrative

Aquaculture is a rapidly growing activity in Minnesota with excellent potential for future expansion as a significant commercial industry. Although faculty at the University of Minnesota have played a leading role in the development of aquaculture in the mid-west through research, extension, and classroom education, efforts to conduct research and extension/demonstration projects are limited by lack of adequate facilities.

Over the past 3 years, the Minnesota Transgenic Fish Group has inserted genes conferring growth enhancement into Minnesota gamefish (trout, walleye, northern pike, salmon). Extensive analysis must continue on these fish and their offspring, the largest group of transgenic fish in North America, testing for level of expression of the genes, their growth effects, and stable transmission to succeeding generations. New strains of transgenic fish are to be developed seeking more optimal expression and growth.

This proposal provides a means of providing adequate facilities for aquaculture/fisheries research and demonstration and for holding genetically engineered fish and continues analysis and improvement of genetically engineered fish.

III. Objectives

A. Construct an aquaculture/fisheries research and demonstration facility on St. Paul Campus of the University of Minnesota.

A.1. Narrative: Construction of this facility will enable University faculty to conduct applied research, demonstration projects, and workshops and will provide space to hold the genetically engineered fish from Objective B of this project as well as from previously funded LCMR projects.

A.2. Procedures: Preliminary plans and estimates have been developed by University staff and a consultant will be hired to confirm the final design. In order to conserve water and energy the facility will be operated largely as a closed system facility. Blowdown water will be discharged to the storm sewer after first passing through a killing field to ensure the destruction of living organisms. Water will most likely be obtained from two existing 800 foot deep wells that were drilled for the now terminated Aquathermal project, previously funded by LCMR. Design and construction contracts will be awarded following appropriate University bidding and contracting procedures.

A.3. Budget

	LCMR Funds
a. Amount Budgeted:	$1,004,000
b. Expenses:	$1,004,000
c. Balance:	$0

A.4. Timeline for Products/Tasks

	Jul91	Jan92	Jun92	Jan93	~~Jun~~ Dec93
Marine appraisals	********				
St. Paul Campus Planning		******			
Consultant hiring			***		
Program development			****		
Design				******	
Construction					**********
Final report					*****

A.5. Status: WORK PROGRAM AMENDMENT
After approval by the Commission of the work plan change in order to build the facility on St. Paul Campus, a consultant was hired to develop the design. The consultant, J.M. Montgomery

1991 - 86

Engineering in association with Lambert and Beck Architects, along with the University planning committee completed program planning in late July. As part of the planning evaluation we compared the cost of putting the facility in a new building versus remodeling a portion of the Agricultural Engineering Shops building. The latter proved to be more cost effective, and thus the facility will be located in the existing building. From late July until late October, the consultant and the University negotiated the final contract terms. The consultant and the planning committee finalized the design in late October. Because of numerous unavoidable delays (see strike throughs below) the LCMR approved an extension of the completion date to December 31, 1993. ~~construction drawings will be completed by mid-December, and the bidding process to hire a contractor for the construction will begin in early January. A few of the major equipment purchases are now out on bids.~~ The first set of blueprints were submitted by the consultant on January 14, 1993, somewhat behind schedule, and they were deemed totally inadequate by U. of M. Facilities Management. Review comments (31 pages) were returned to the consultant on February 10. The second set of blueprints were received on March 3, slightly improved, but still inadequate. Review comments (26 pages) were returned on March 29. The third set of blueprints were received on April 22 and these were good except for the need to redesign a door which delayed the approval by the University for 2 weeks until June 3. Advertisement for a construction contractor appeared in the Construction Bulletin on June 11 and 18 and bids will be opened on July 1. With the approval of the blueprints the funds have been encumbered for construction but not liquidated. The contract requires 150 days for completion, so if all goes well, the facility will be finished by mid-December 1993. The $44,000 that was transferred from Objective B to A after approval of the LCMR has been encumbered for the purchase of tanks. Bids from potential vendors will be opened on June 25.

~~According to University staff and J.M. Montgomery, Consulting Engineers Inc., the project should be completed sometime in September 1993. Knowing that unforeseen delays frequently occur during major construction projects, the project completion date has been extended to December 31, 1993. The reasons for the no cost extension are as follows:~~

~~1) The first 7 months of the project were spent evaluating the purchase of the Marine Minnow Farm and concluding that it was not a viable alternative. The current work plan, to build on St. Paul Campus, was not undertaken until after the Commission approved the work plan change on February 6, 1993. Most of the approximately $10,000 that have been spent from the budget thus far were for appraisal fees and water analysis related to the Marine Minnow Farm evaluation.~~

~~2) Because of state rules for bidding and the need to go through the State Designer Selection Board, a consultant was not hired until after May 11, 1993. Everyone cooperated to the maximum to keep this process on the fast track, but rules for timing of the bidding process and the normal difficulty in scheduling meetings among numerous participants prevented any quicker action.~~

~~3) There have been a number of delays in the University's negotiating the contracts with J.M. Montgomery which were completely out of the control of the project manager. According to University staff, the consultant was very slow to respond to proposals made by the University.~~

~~4) Now that the contract with the consultant current work plan is underway, the realistic time it will take for design completion, letting a contract for bids, hiring the contractor, purchasing equipment, and actual construction is likely to be a little less than a year. LCMR funds should all be encumbered by July 1993.~~

~~Since the final product will be a functioning facility, the LCMR will be able to clearly demonstrate the results of their expenditure.~~

A.6. Benefits: Construction of the facility will provide a state of the art facility for applied aquaculture/fisheries research, demonstrations, and workshops and will provide adequate space for holding the genetically engineered fish. Faculty at the University will be able to take advantage of funding sources for these kinds of projects from such organizations as the North Central Regional Aquaculture Center, Greater Minnesota Corporation, USDA, Minnesota DNR and the Fish and Wildlife Service. The previous investment in research of approximately $1,000,000 to develop the existing group of transgenic fish will be preserved.

B. **Continued analysis (growth, performance, molecular, and breeding) of transgenic fish; ~~creation of new transgenic fish to test new genes.~~**

B.1. Narrative: Extensive analysis must continue on the current transgenic fish, which are a resource representing approximately $1,000,000 in research funding. Analysis of growth potential, transgene effect, and inheritance of the transgene is required. Continuation of effort is needed to isolate other growth promoting fish genes and their controlling elements, with subsequent transfer into fish, establishing new transgenic fish lines. Transgenic fish will not be released into the wild in the foreseeable future, however, ecological studies addressing this important issue are presently in the planning stage.

B.2. Procedures: **DNA analysis**. The presence and physical state of transgene DNA within existing transgenic fish is characterized by Southern blot hybridization analysis. Isolated fin DNA from juvenile fish (Hallerman et al. 1990: by sodium dodecyl sulfate lysis (SDS) and proteinase K digestion) is digested with excess BamHI restriction endonuclease, electophoresed through a 0.8% agarose gel, transferred onto nylon membranes by Southern blotting and then probed for the presence of transgenic DNA. Probes used to identify positive fish consist of either pRSV/bGH, pRSB/npGH or FV-2/csGH labeled with ^{32}P by the random priming method.
RNA analysis. The presence of transgene poly (A)+ RNA (Glisin, et al. 1978) will be analyzed by dot-blot hybridization employing radioactive probes labeled to high specific activities (ca. 5 x 10^8cpm/ug) to determine whether any quantitative differences exist between various cell and tissue types in a given transgenic fish, and by Northern blot hybridization (Thomas, 1980) to determine if any qualitative differences can be detected. In situ hybridization

will be used to analyze both whole sections of embryos and cross sections of juvenile fish to identify the stage in embryogenesis as well as the specific tissues type in which transgene related sequences are being expressed (Haase et al. 1984). This technique offers several advantages over the standard methods of hybridization including: 1) very small tissues samples can be used directly without RNA extraction and purification; 2) the sensitivity of this technique will allow the detection of a minimal amount of transcripts from just a few cells in a tissue preparation (an extremely important issue for mosaic founder fish); and 3) most importantly, expression can be localized in tissues thereby providing information about the specific types of cells within a tissue sample that are expressing specific transgenic sequences. Briefly, sections from formaldehyde-fixed and paraffin-embedded tissues are attached with the aid of an adhesive to microscope slides that have been acid washed, treated with Denhardt's solutions and the acetylated. Following deparaffination with xylene and dehydration, sections are acid extracted, neutralized, and treated sequentially with digitonen, proteinase K, and DNase. Large samples can be serially examined while frozen in carboxy methyl cellulose by sectioning with a cryostat. Tritium-labeled DNA probes are hybridized for two days at room temperature in formamide-destran sulfate solutions. Following extensive washing, slides are covered with photographic emulsion and developed at appropriate times. Positive hybridization is selected by either light of electron microscopy following hematoxylin staining as evidenced by an excess of silver grains over cells as compared to control samples.

Growth hormone radioimmunoassay. Blood scrum samples collected from dorsal aortae of two month old fish will be analyzed for the presence of transgene GH protein by radioimmunoassay. Because the growth hormone containing constructs include the signal peptide sequence (responsible for secretion of the hormone into the blood stream under normal circumstances), detection of transgene GH protein is expected. Radioiodinated transgene GTH is prepared by adding four iodo-Beads (Pierce Chemical Co., Rockford, IL) to a vial containing 1.5 mCi ^{125}I and 0.2 ml buffer (0.25 M Na_3PO_4 pH 7.5). After 5 minutes, 14 mg bovine growth hormone (USDA) solubilized in 0.01 M. $NaHCO_3$ diluted with 20 ml of buffer is added and incubated for 15 min. The mixture is purified by column chromatography using Bio-gel P-30 (Bio-Rad) equilibrated and eluted with 0.1% bovine serum albumin (BSA)-barbitol buffer pH (Sigma) as three peaks. The middle peak is diluted in 1% BAS-0101 phosphate-buffered saline M. (PBS) pH 7.5. Anti-serum to ovine GH (NIAMDD-anti-oGH) is diluted 1:5,000 in 1% normal rabbit serum-.05 M EDTA-PBS pH 7.0, and 0.1 ml dispensed into assay tube containing 0.3 ml 1% egg albumin-PBS pH 7.0 and 0.32 ml test plasma. After 24 hours later by the addition of 0.1 ml precipitating antiserum (sheep anti-rabbit gamma globulin) and incubation for 48 hours. Samples are centrifuged at 1,500 X g for 20 min and counted in a Beckman gamma counter. Assay sensitivity is approximately 1 ng/tube or approximately 1 ng/ml (Wheaton et al., 1986).

Immunohistochemical detection. Fin tissue collected from individual fish in the microinjected groups can be fixed, sectioned, and mounted on slides using standard clinical techniques. Slides are deparaffinized by a 30 min room temperature dip in xylol, followed by two 5 minute dips in 100%, 90%, and 70% ethanol. Slides are washed three times in PBS pH 7.6 for ten minutes and in PBS for five minutes. Blocking is by incubation in a 1X dilution of normal sheep serum for 30 minutes at 37°C, followed by a gentle wiping. Slides are incubated in polyclonal rabbit anti-bGH diluted 1:100 in normal sheep serum overnight at 4°C, washed three times in PBS for 10 minutes, and incubated with goat anti-rabbit Ig diluted 1:2,000 in normal sheep serum for 30 min at room temperature. Slides are washed three times in PBS for 10 min, and incubated with the DAB (Diaminobenzidine tetrahydrochloride) substrate (5mg DAB in 20 ml 0.03% H_2O_2 in PBS) for 10 min at room temperature in the dark. Slides are washed three times in PBS, counterstained in 0.15% methylene blue for 10 min, washed twice in tap water, dehydrated by a series of dips in 70%, 95%, and 100% ethanol, dipped in xylol twice, and mounted with Permount. growth of selected fish is monitored monthly for weight and length grain.

B.3. Budget

	LCMR Funds
a. Amount Budgeted:	$196,000
b. Expenses	$170,034
b. Balance:	$0

B.4. Timeline for Products/Tasks

	Jul91	Jan92	Jun92	Jan93	Jun93
Analyze existing transgenic fish	**********				
Breed transgenic fish at maturity	**********	**********	**********		
Analyze offspring			**********	**********	
Growth studies of offspring			**********	**********	
Analyze breeding data				*****	
Designate broodstock				*****	

B.5. Status: WORK PROGRAM AMENDMENT

1. Current Status of Transgenic Fish

a) Northern Pike:

1988 Founder stock (RSVbGH): 36 founder fish remain from the 1988 founder stock (7 males and 29 females), including all five previously identified as positive for the transgenic Growth Hormone (GH) gene construct. 24 control siblings are also alive. Growth studies have indicated an enhancement effect of the extra growth hormone gene in some cases. One two-year old (1990) cross exists as of 6/93, however only 2/25 remaining siblings contain an extra growth hormone gene (other positive siblings died). These positive fish have not demonstrated enhanced growth, however rearing conditions have been much less than ideal. In 1991, 3/8 crosses of the 1988 stock were positive for the transgenic GH gene by Southern blot analysis. Only 12 fish remain from these crosses. Many of the remaining

1988 founder fish spawned during the month of May 1993: 9 males were mated with 15 females to yield 23 crosses (2 of these are control sibling crosses). As of 6/93, 18 crosses have had various degrees of success hatching. These crosses will be screened for transmittance of transgene in ~June/July, 1993.

1989 Founder stock: In April 1991 due to limited space on the St. Paul campus, these fish were split and moved to hatcheries in Cohasset and New London, MN. The Cohasset fish all died from a facility malfunction. The New London fish went through strong negative selection due to health problems (well water on the St. Paul campus is from an aquifer and thus somewhat sterile; whereas the NL water was taken from the bottom of a outdoor pond and thus contains all of the parisites a fish in the wild may succumb to, winter water temperatures was only 39`F). Only the fittest fish survived, which somewhat surprisely did not include the known transgenic founder fish. Only 54 of the ~200 fish (>50% known to be transgenic) that were moved to NL returned to the St. Paul Campus, and only 2 of these were known to carry transgenic GH gene from blood and tissue assays by Southern blotting. As of 6/93 only 33 fish survive, with only one of the known transgenic founders being among them. However, this female is the fourth largest amongst its siblings. The other 32 are potential mosaics; they are not positive in fin and blood samples but may be positive in their gonads. Consequently, these fish are being kept until gametes can be obtained and screened for transgenic GH gene. The remaining 1989 founders did not produce gametes in 1993.

b) Rainbow trout:

1989 Founder stock with the bGH gene: No founders have survived. 25 crosses were made in 1990. 6 of 14 crosses tested at the embryo stage were found, by Southern blotting of DNA from selected samples of embryos, to be positive for extra bGH genes. However, when checked again after survival to the fingerling stage, only two crosses still had positive offspring, as of 6/93 there are 45 siblings alive of which only 3 possess transgenes. There is no apparent growth enhancement in these fish.

1990 Founder stock with the csGH gene: The health of these fish deteriorated as the 1993 spawning season approached. 39 fish died during this stressful period. The reason for these deaths is not understood, but it also affected gamete quality in the remaining living fish. Only 4 females gave eggs of sufficient quality to be fertilized during Febuary and March of 1993. Different males were used to ferlize these eggs to yield 9 crosses. 3 of these hatched and are presently being screened for transgene transmittance. Two of these crosses have ~1000 siblings each, the third cross only has 26 siblings. Of the remaining 61 founder stock, 12 fish are still alive that are known to be positive for extra csGH genes. Fortunately, all of the fish known to transmit the extra csGH genes are still alive. The other founders are all potentially mosiac and thus are potentially capable of transgene transmittance (when these fish eventually breed we will be able to determine if transmittance is possible). As mentioned in previous updates, all control founder siblings (mock injected, non-transgenic fish) were sacrificed due to space limitations thus destroying any ability to determine growth enhancement in the founder stock. There are three positive crosses from the 1992 mating season that were screened for transgene transmittance (~400 fish) in May 1993. Only one of these appears to have any positive offspring remaining; frequency ~1% for transmittance.

c) Atlantic salmon:

1990 Founder stock with the csGH gene: As of 6/93, 118 founder fish are surviving; 5 of these are known to be positive for transgenic csGH gene within their fin tissue. The remaining fish are potentially mosiac, and thus have a chance of having transgenic gametes. 20 early-maturing female fish were bred in late 1992; 21 crosses were made of which 17 were successfully fertilized. Unfortunately, most of the embryos died shortly before hatching. There are ~130 fish remaining alive from 8 of these crosses. The low survivability might be due to the early maturation of the gametes, a full year early. Whether the transgene was passed on in these crosses is unknown as of yet. 25 males produced gametes, however, analysis of the sperm DNA showed only 1 possessed the transgene. The growth performance of the atlantic salmon founder fish is impossible to evaluate since all control siblings have been sacrificed due to space limitations. Nearly all founders should spawn in 1994, and the gametes are expected to be of high quality.

d) Walleye:

1990 Founder stock with the csGH gene: All have died. No progeny were obtained before death..

e) Summary:

Although the number of surviving founder fish is low, many are apparently transgenic. Only one or two actively expressing fish are required to serve as broodstock for future generations. The outlook positive.

2. Current Status of Transgenic Fish Culture

Owing to the requirements that the transgenic fish be maintained indoors, we have had to move the fish to several hatcheries throughout the state of Minnesota. These moves, accompanied with occasional periods of poisoning with chlorinated water and high/low temperature water, severely stressed the potentially transgenic fish. Consequently, with financing obtained from the University of Minnesota/Legislative Commission of Minnesota's Resources, a new facility is being constructed that will house the products of

this project. This should i) eliminate the problems we have encountered in maintaining adult fish to reproductive age, and ii) provide adequate space to determine the enhanced growth of second generation fish.

We continually breed the transgenic fish but currently have to discard older fish to continue new lines. If the new facility is constructed soon enough, we will be able to raise the offspring of atlantic salmon mated in December 1992, the rainbow trout offspring from March 1993 matings, and the northern pike offspring from May 1993 matings. This would provide us with a large number of potential broodstock for behavioral and performance analysis of transgenic fish by Prof. Kapuscinski.

3. General observations on transgenic fish.

Although these studies were not designed to evaluate the overall fitness of fish transgenic for growth hormone genes, preliminary evidence strongly suggess that their fitness is low. Several lines of evidence supports this:

i) Mendelian inheritance of transgenes would be expected to be ~>/= 50%, however when hatchlings have been screened we have yet to see more than 20% transmittance (usually ~5-10%). This suggests that selection is occuring during embryo maturation that preferentially effects the transgene carrying siblings.

ii) in every documented case to date, once a positive cross has been identified (shortly after hatching), by the time these fish are one year of age most of the siblings possessing the transgene have died. Again suggesting a low fitness value for those possessing transgenes.

iii) perhaps the most convincing evidence is the lack of survival by transgenic fish moved to the New London facility. These fish went from the near sterile environment at the St. Paul Campus to an environment filled with every normal pathogen a wild fish might encounter (water was taken from the bottom of a outdoor pond). Of the 200 fish that were moved there, 49 were known to be transgenic. Only 2/54 returning fish were carriers. Today only one of positive fish survive suggesting that their over all fitness did not match their nontransgenic co-siblings (some of the survivers might be mosiac though).

This evidence might suggest that other less pleiotrophic growth enhancing genes should be used in place of GH genes.

~~4. Current Status of Gene Cloning.~~

~~We do not have facilities to house new transgenic fish. When this proposal was submitted, we anticipated that as we made transgenic fish, we would either 1) move them to outstate hatcheries for breeding or 2) release the fish into outside facilities used by the MN DNR for evaluation. However, we have found that movement of transgenic fish from one facility to another in the past three years induces considerable stress to these animals and this stress was a major contributor to the loss of nearly 80% of our transgenic stocks. Moreover, in the past year the state has re-examined the issue of release of genetically engineered organisms and EQB has adopted rules which are more stringent than anticipated. In order to put our transgenic fish into outdoor facilities, we will need to obtain more information on their feeding and behavior. These experiments are currently being designed. In the meantime, the fish must remain indoors. Our abilities to keep fish has been curtailed because as the fish increase in size, the number per tank must be lowered. As a result, we are constantly culling fish that are not transgenic. Without keeping non-transgenic fish (controls), we cannot do growth studies.~~

~~Consequently, we cannot keep the fish we have alive and at the same time create new lines of fish. We have the vectors and the genes, and through federal funding obtained on the basis of the LCMR results to date, we are continuing to develop new growth enhancing genetic constructs. In particular, a U.S. Department of Agriculture grant is funding investigations into alternative growth stimulating genes, like the c-ski gene which is specific for muscle tissue and therefore lacks some of the unsettling aspects of more global hormones like growth hormone. But, we need expanded facilities for these fish and until we have them, we cannot produce more fish.~~

~~Accordingly, we request that the $44,000 budgeted for the development of new lines of transgenic fish be used instead to purchase tanks to hold both the growing offspring of our current transgenic fish and the fish we want to produce but have no room for at present. The cost for construction of the new facility (Objective A) has left no money for the purchase of large tanks for transgenic fish, but there is space for the tanks. The fish we proposed to create will be produced, using the money from the USDA grant ($250,000), mentioned in the previous paragraph, and both the old and the new fish will be maintained in the new facility if the tanks become available. Thus, we think it best in terms of the goals of the LCMR program to redirect the funding originally targeted for more transgenic fish to the purchase of tanks to maintain the valuable fish we already have and those we planned to make.~~

B.6. Benefits:

a. Determination of economic potential of some 2000 existing transgenic fish (walleye, northern pike, salmon, rainbow trout);
b. Develop broodstock of optimal transgenic fish for research and possible commercial application;
c. Establish breeding protocols for maximal analysis of future transgenic fish;
d. Possible finer control over growth enhancement through use of other growth promoting genes [e.g. insulin-like growth factor (IGF-1), growth hormone releasing factor (GHRF)];
e. Isolation of growth promoting genes for possible expression and use of product as feed supplement;
f. Creation of new lines of transgenic fish to complement growth hormone lines in existence.

IV. Evaluation:

For the FY 92-93 biennium the program can be evaluated by: 1) successful construction of a state of the art aquaculture/fisheries research and demonstration facility 2) identification and spawning of the transgenic fish showing the best growth enhancement potential, 3) characterization of the status of the transgene in expressing fish, and 4) successful injection of new constructs containing fish growth hormone genes in the appropriate species. Long term evaluation of the project's success will be determined by future success in conducting applied research, demonstrations, and workshops at the facility to benefit the development of aquaculture in Minnesota and in developing broodstocks of transgenic fish with enhanced growth characteristics.

V. Context:

A. Objective A---The existing laboratory on St. Paul Campus is lacking both water flow capacity and space. When that laboratory was completed in 1969, research was largely confined to working with small species or young fish, mostly in pollution related studies. In recent years, emphasis in research and teaching has shifted to large species in sports fishing or aquaculture situations.

The rapid growth of interest in aquaculture has provided new opportunities for research and extension that cannot be pursued in the existing facility. The recently established North Central Regional Aquaculture Center has provided a new source of funds for aquaculture research and technology transfer. Although University faculty are participating and even leading the way in much of the Center's activities, only a small portion of the Center's funding has been awarded to the University because facilities are inadequate. Approximately $1.35 million in total research funding has been lost over the last 3 years as a result of not having adequate facilities.

In comparison to Minnesota, most state universities in this region have much more extensive facilities, generally built or upgraded in the last 5 years. Although some of the research done at these locations will be applicable to Minnesota, the more local concerns will not receive adequate attention, and Minnesota will fall behind in its attempt to develop an aquaculture industry.

Objective B---Over the past 4 years, the Minnesota Transgenic Fish Group has established itself as one of the most active fish genetic engineering groups in the world and has initiated a large effort in the economic improvement of fish via genetic engineering, with primary focus on the introduction of extra growth promoting genes by gene transfer techniques. Currently, about 3000 transgenic fish are being maintained on the University campus (Hodson Hall) and off campus (DNR, MN Power). These fish, of four species (northern pike, walleye, salmon, and rainbow trout), are being cultured to determine the efficiency of the transfer and growth enhancement, to study the biology of such transgenic organisms, and to mature for breeding to determine inheritance of the trait and to establish strains of these species with improved growth rates. This latter goal necessitates maintenance of the fish for 2-4 years per generation. To date, transgenic fish have been produced using a bovine growth hormone gene and viral promoters into salmon, pike, trout, and walleye, and by inserting a salmon growth hormone gene into salmon and pike. Our ideal transgenic fish, however, contains only pieces of added DNA that originally came from that species, i.e. an added northern pike gene into northern pike. The proper transfer system and vector has been produced by our group, and the northern pike and walleye growth hormone genes have been isolated and cloned. This step is necessary to meet regulatory concerns for human consumption of these fish and to establish the proper strain of recombinant fish for ecological studies.

Thus current work has produced a large number of transgenic fish which must be further analyzed to determine the effectiveness of the transferred gene and its passage frequency to future generations. The production of the ideal transgenic fish has been begun by inserting the salmon gene, however these fish also must be analyzed and bred, requiring culture until sexual maturity. The production of transgenic pike and walleye using the species specific gene needs to be begun, and all components (vector, methodology, and cloned genes) are in place. This will enable the production of optimally useful transgenic fish for commercialization when current questions concerning dangers of genetic-engineered fish are resolved.

B. Objective A---Construction of the aquaculture/fisheries research and demonstration facility will provide the kind of facility needed to advance aquaculture in Minnesota. Construction of this facility will provide a state-of-the-art aquaculture/fisheries research and demonstration site.

Objective B---The proposed work will add to existing data and work in progress by enabling the complete analysis of existing fish, allowing the maturation and breeding of the existing fish, and by producing better transgenic fish (northern pike and walleye) using existing methods and genes. This supplementary work will enable the better selection of transgenic fish for broodstock generation, by analysis of the levels of expression of the transgene in existing fish, and examination of the stability and state of the transgene DNA in the fish. Since each individual fish in the injected population takes up the transgene a little differently, a search of the whole population for the best uptake and function of the gene is necessary. This in turn gives data enabling better success in the future. The proposed work also entails creation of two new populations of transgenic fish, northern pike with an added northern pike growth hormone gene and walleye with added walleye growth hormone genes, both to verify the data gained in these species using a cow growth gene and to establish strains of transgenic fish suitable for commercial development.

C. Objective A---The Department of Fisheries and Wildlife, University of Minnesota has had a long history of conducting pioneering and internationally recognized research in aquaculture and fisheries including two aquaculture projects funded by LCMR (Genetic Engineering of Minnesota Fishes and Improvement of Pond Aquaculture in Minnesota). It is likely that there will be future proposals to LCMR to conduct specific research or demonstration projects at the new facility.

Objective B---The Transgenic Fish Project was begun under Sea Grant (Federal) funding which enabled the initial development of transfer techniques. Other non-LCMR funds (Blandin Foundation

and Greater Minnesota Corporation) have supported much of the work with salmon and trout, creating transgenic rainbow trout with a bovine growth hormone gene that are now just old enough to be bred. Indeed one of the precocious male transgenic trout has shown the gene to be passed on to the next generation. Sea Grant funding has continued to members of the group and has allowed investigation to begin on other growth promoting genes and the possibility of transfer of disease resistance. Sea Grant funding has also aided in the construction of the all-fish expression vector which has been and will be used in producing ideal transgenic strains. Non-LCMR funds have totaled about $820,000 over the past 6 years. The investment has spawned: 1) U.S. Department of Agriculture funding of investigations into alternative growth stimulating genes ($250,000), 2) National Institute of Health funding of alternative model fish systems for testing hormone action, *e.g.*, thyroid hormone receptor activity in zebrafish ($450,000), and Sea Grant funding for additional research on transfer of the growth hormone gene ($120,000).

The LCMR funded the development of the transgenic process for northern pike and walleye and the start of the transgenic population of these fish containing the bovine growth hormone genes. The LCMR money also enabled the cloning of the northern pike and walleye growth hormone genes, and aided in the construction of the expression vector to be used with them. The LCMR funds (1987-89) were critical to getting the project well established and have permitted it to be developed into one of the premier such efforts in the world.

Potential future LCMR proposals may be considered to examine the ecological interaction of wild and transgenic growth-enhanced fish and to extend the technology to solve other genetic-related problems (disease resistance, cold or heat tolerance, etc.).

D. Not applicable.

E. Biennial Budget System Program Title and Budget: Not Available at this Time

VI. Qualifications

1. Program Manager:
   Dr. Ira R. Adelman
   Professor and Head
   Department of Fisheries and Wildlife, University of Minnesota

   Ph.D. Fisheries, University of Minnesota, 1969

   Dr. Adelman has conducted research on the environmental physiology of fishes for over 20 years. Much of that research has been applicable to aquaculture. He is on the research committee of the North Central Regional Aquaculture Center and on the Minnesota Aquaculture Commission. As head of the Department of Fisheries and Wildlife he has been responsible for administration of department programs and a budget as high as $3 million annually including external grants. Dr. Adelman's primary role will be as program coordinator and to oversee work conducted under Objective A.

2. Major Cooperators:

   A. Dr. Anne R. Kapuscinski
      Associate Professor and Extension Specialist
      Department of Fisheries and Wildlife and Minnesota Sea Grant College, University of Minnesota

      Ph.D. Fisheries/Genetics, Oregon State University, 1984
      M.S. Fisheries/Aquaculture, Oregon State University, 1980

      Dr. Kapuscinski has conducted extensive research in aquaculture and fish genetics and has been responsible for an extension program aimed toward the development of aquaculture in Minnesota. In working with farmers and regulatory agencies, she is familiar with the problems associated with effluents from aquaculture facilities and in control systems. Dr. Kapuscinski will be responsible for assisting Dr. Adelman in the completion of Objective A.

   B. Dr. Anthony Faras
      Professor
      Department of Microbiology, University of Minnesota

      Ph.D. Pathology/Virology, University of Colorado Medical Center, 1970

      Dr. Faras's primary expertise is in the areas of viral molecular biology and analysis and the molecular cloning of genes. While most of his work has been in human systems, over the past few years, he has expanded into other animal systems with a special emphasis on fish. He has been a member of the Minnesota Transgenic Fish Group since its inception and has been responsible for the cloning of walleye and northern pike growth hormone genes for use in the group project. Dr. Faras's primary role will be to continue the cloning efforts and perform molecular analysis on the transgenic fish under Objective B.

   C. Dr. Perry Hackett
      Professor
      Department of Genetics and Cell Biology, University of Minnesota

      Ph.D. Biophysics/Genetics, University of Colorado Medical Center, 1974
      M.S. Biophysics/Genetics, University of Colorado Medical Center, 1970

Dr. Hackett has been a member of the Minnesota Transgenic Fish Group since its inception and has been responsible for the development of an all-fish expression vector for use in transferring genes. He and his group are involved in the genetic manipulations needed to permit optimal expression of the cloned genes in the transgenic fish. Dr. Hackett will continue his vector construction and analysis, seeking to improve the vector for use under Objective B.

3. Scientific Reports Published or in Press since the inception of the grant:

1) Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1991). Importance of the *CArG* box in β-actin gene expression. Gene 108:211-217..

2) Gross, M.L., Kapuscinski, A.R. ,Schneider, J.F., Liu, Z., Moav, N., Moav, B., Myster, S.H., Hew, C., Guise, K.S., Hackett, P.B. and Faras, A.J. (1992). Growth evaluation of northern pike(*Esox lucius*) injected with growth hormone genes. Aquaculture 103: 253-73.

3) Schneider, J.F., S.H. Myster, P.B. Hackett, K.S. Guise, and A.J. Faras (1992). Molecular cloning and sequence analysis of the cDNA for northern pike (*Esox lucius*) growth hormone gene. Mol. Mar. Biol. Biotech. 1: 106-112.

4) He, L., Z. Zhu, A.J. Faras, K.S. Guise, P.B. Hackett, and A.R. Kapuscinski (1992). Characterization of AluI repeats of zebrafish (*Brachydanio rerio*). Mol. Mar. Biol. Biotech. 1: 125-135.

5) Moav, B., Z. Liu, Y. Groll, and P.B. Hackett (1992). Selection of promoters for transgenic fish. Mol. Mar. Biol. Biotech. 1: 338-345.

6) Moav, B., Z. Liu, N.L. Moav, M.L. Gross, A.R. Kapuscinski, A.J. Faras, K. Guise, and P.B. Hackett (1992). Expression of heterologous genes in transgenic fish. IN: *Transgenic Fish* (C.L. Hew and G.L. Fletcher, eds.) World Scientific Pub. Co., pp. 120-141.

7) K. Guise, P.B. Hackett, and A.J. Faras (1992). Transfer of genes encoding neomycin resistance, chloramphenicol acetyl transferase and growth hormone into goldfish and northern pike. IN: *Transgenic Fish* (C.L. Hew and G.L. Fletcher, eds.) World Scientific Pub. Co., pp. 120-141.

8) Hackett, P.B. (1992). The molecular biology of transgenic fish. IN: *Biochemistry and Molecular Biology of Fishes*. (P. Hochachka and T, Mommsen, eds.) in press.

9) Moav, B., Z. Liu, L. Caldovic, A.J. Faras, and P.B. Hackett (1993). Regulation of early expression of transgenes in developing fish. Transgenic Research 1: 153-161.

10) Ivics, Z., Z. Izsvak, and P.B. Hackett (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Mol. Mar. Biol. Biotech. 2:162-173.

11) Caldovic, L., S. Fahrenkrug, J. Breuer, and P.B. Hackett ( ). Piscine expression vectors with polycloning sites. in preparation.

VII. Reporting Requirements

Semiannual status reports will be submitted not later than January 1, 1992, July 1, 1992, January 1, 1993, and a final status report by June 30, 1993 for Objective B and December 31, 1993 for Objective A.